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Chimeric drift and immunological tolerance in allophenic mice

by

Thomas J. Stephens

A Dissertation Submitted to the
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DOCTOR OF PHILOSOPHY

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LIST OF ABBREVIATIONS AND SYMBOLS

α chain	alpha chain of hemoglobin
Anti-RS	anti-recognition structure
B cell	bursa-equivalent derived cell
β chain	beta chain of hemoglobin
β^{dmaj}	diffuse (double) hemoglobin beta chain major component
β^{dmin}	diffuse (double) hemoglobin beta chain minor component
β^{pmaj}	partial diffuse (double) hemoglobin beta chain major component
β^{pmin}	partial diffuse (double) hemoglobin beta chain minor component
β^{s}	single hemoglobin beta chain
BSA	bovine serum albumin
C	complement
CPM	counts per minute
CML	cell mediated lympholysis
Con A	concanavalin A
CRNA	carrier ribonucleic acid
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
$DNP_{56}BGG$	2,4 dinitrophenyl bovine gamma globulin
$DNP\text{-}GLA^{5}$	2,4 dinitrophenyl L-glutamic^{57}, L tyrosine^{38}, L-alanine^{5}
DOC	deoxycholate
EDTA	ethylenediaminetetraacetate

EEA	early embryo aggregation derived chimeras
Fc	fraction crystallizable
FCS	heat-inactivated fetal calf serum
F-H	Ficoll-Hypaque solution
(GAT^{10})	L-glutamic acid^{60}, L-alanine^{30}$_m$ K-tyrosine^{10}
CPI	glucose phosphate isomerase
GVH	graft versus host response
H-2	histocompatibility-2 complex
[^{3}H]uridine	tritiated uridine
Hb	hemoglobin
Hbb^d	diffuse (double) hemoglobin
Hbb^p	partial diffuse (double) hemoglobin
Hbb^s	single hemoglobin
hCG	human chorionic gonadotrophin
IDH	isocitrate dehydrogenase
Ig	immunoglobulin
Ir gene	immune response gene
IU	International Unit
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LCM	lymphocytic choriomeningitis virus
Ly	lymphocyte differentiation antigen
M	molar
MASH	multiple automated sample harvester
2-ME	2-mercaptoethanol
mCi	milliCurie

MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
PAGIF	polyacrylamide gel isoelectric focusing
PBS	phosphate buffered saline
PHA	phytohemagglutinin
(Phe,G)-A--L	(poly-L-phenylalanine, L-glutamic acid)-polyD,L-alanine--poly-L-lysine)
pI	isoelectric point
PMS	pregnant mare serum
POPOP	P-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5 diphenyloxazole
psig	pressure per square inch
PVP	polyvinylpyrolidone
PWBC	peripheral white blood cell
resp.	response
RNA	ribonucleic acid
S.D.	standard deviation
SWBC	spleen white blood cell
T cell	thymus derived cell
TCA	trichloroacetic acid
TEMED	N,N,N^1,N^1-tetramethylethylenediamine
Thy	thymus antigen
(T,G)-A--L	(poly-L tyrosine, L-glutamic acid)-poly D,L-alanine--poly L-lysine
(T,G)-P--L	(poly-L-tyrosine, L-glutamic acid)-poly-L-proline--poly L-lysine
Tla	thymus leukemia antigen

TNP	2,4,6 trinitrophenyl
TWBC	thymus white blood cell
μCi	microCurie
V genes	variable region genes
v/v	volume/volume
w/v	weight/volume

I. INTRODUCTION

A. What Are Allophenic Mice?

Allophenic mice are chimeras. The term chimera is used to describe a composite plant or animal in which the different cellular populations are derived from more than one fertilized egg or the union of more than two gamates (McLaren, 1976). Plants or animals in which the tissues are combined from two or more adult individuals or from embryos after the period of organogenesis are referred to as secondary chimeras while primary chimeras are individuals in which genetically different cell populations coexist from a very early stage of embryogenesis (Ford, 1969; McLaren, 1976). A different term, mosaic, is used to describe a composite individual derived from a single fertilized egg (McLaren, 1976). Secondary chimeras are chimeric in only one tissue or organ. On the other hand, all primary chimeras may be chimeric in any or all of their tissues or organs. Allophenic mice are primary chimeras. The preparation of these mice was first described by Tarkowski (1961) and shortly thereafter modified by Mintz (1962a,b). In the Mintz procedure, allophenic mice are formed by the aggregation of two embryos at the 8-cell stage of development subsequent to removal of the zona pellucida by pronase. After 24-36 hours in culture, the double-sized blastocysts are transferred to a pseudopregnant foster female and allowed to come to term. Allophenic mice derived from the aggregation of two embryos are designated as strain 1 ⟷ strain 2 mice.

An alternate method for making allophenic mice is the injection technique (Gardner, 1968). In this technique, cells from a dissociated blastocyst are injected singly or in groups into the blastocoele of an intact blastocyst and become incorporated into the inner cell mass of the recipient embryo, giving rise to an injection chimera (McLaren, 1976). Allophenic mice derived from the injection procedure are designated strain 1 $\longrightarrow$ strain 2 mice, where strain 1 is the donor and strain 2 is the recipient blastocyst. Both the aggregation and injection techniques of making allophenic mice are shown in Figure 1.

Other terminology is often used synonymously with allophenic mice (Mintz, 1967). These terms include quadriparental (Mintz, 1965), tetraparental (Wegmann and Gilman, 1970), ovum fusion derived (Barnes and Tuffrey, 1972), and early embryo aggregation (EEA) derived chimeras (Barnes, 1976). Allophenic mice have been used extensively as laboratory test systems in the scientific fields of experimental embryology, genetics, and immunology. Some of the markers used to identify the different cell populations in an allophenic mouse include cell surface antigens (Mintz and Palm, 1969; Mintz and Silvers, 1970; Bona et al., 1974; Festenstein et al., 1975; Warner et al., 1976, 1977a,b,c,d; and Stephens et al., 1977), hemoglobin β-chain differences (Wegmann and Gilman, 1970; Stephens et al., 1977; and Warner et al., 1977d), biochemical variations of the enzymes glucose phosphate isomerase (GPI) and isocitrate dehydrogenase (IDH) (Gearhart and Mintz, 1972; Chapman et al., 1972; Mintz and Baker, 1967; and Ansell et al., 1974), and chromosomal markers such as the T6 chromosome translocation (Mystkowska and Tarkowski, 1968; Ford et al., 1975).

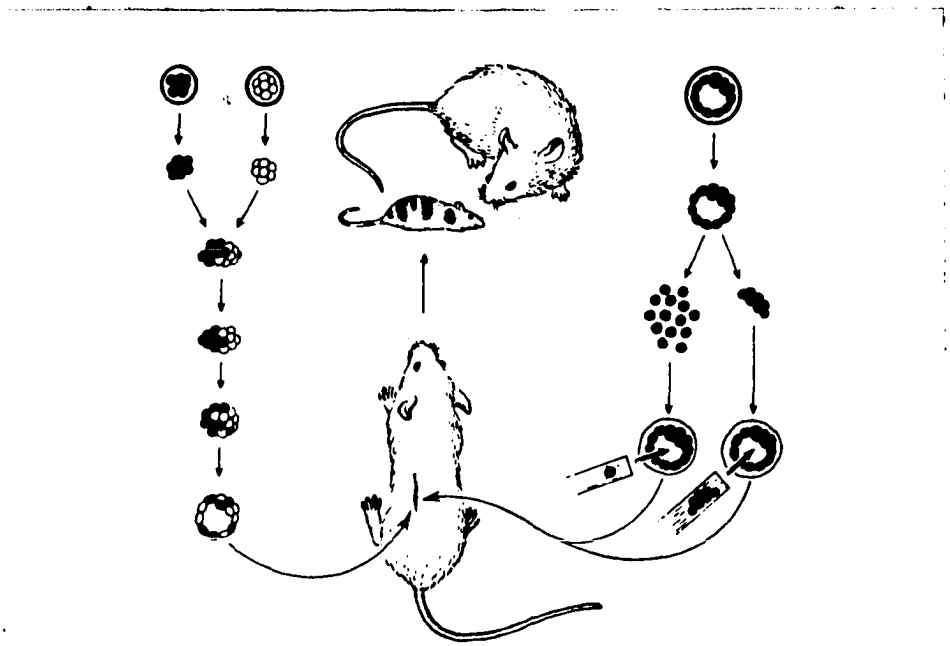


Figure 1. Diagram to show the essential steps involved in making aggregation chimeras (on the left) and injection chimeras (on the right). [For both, the procedures represented in in the outer columns are carried out in vitro (McLaren, 1976).]

B. Phenotypic Sex in Allophenic Mice

Since attempts to sex living embryos at the 8-cell or blastocyst stages of development have not been successful, allophenic mice are synthesized without regard to the sex of the component strains comprising the mice (McLaren, 1976). As a result, allophenic mice tend to distribute themselves into populations consisting of 25% males (XY ⟷ XY), 25% female (XX ⟷ XX) and 50% male or female (XX ⟷ XY). Experiments in chromosomal sexing of mice has consistently agreed with this ratio (Mystokowska and Tarkowski, 1970; Milet et al., 1972; McLaren, 1975; and Ford et al., 1975).

XX ⟷ XY allophenic mice may show varying degrees of dominance of either sex, ranging from 100% development as phenotypic males or females to an equal ratio of males to females in some strain combinations (McLaren, 1976). The expression of this dominance is thought to reflect the relative distribution of XY and XX cells in the primordial gonads (McLaren, 1976). Whether phenotypic sex is determined by the somatic or germ cell components of the gonads is still controversial. Arguing in favor of the somatic cells is the investigation of Ford (1970) who observed that in XO/XY human mosaics the more masculinized gonad is usually characterized by a higher proportion of XY cells in cultures grown from its somatic tissue and that the genital ridge undergoes its initial sexual differentiation even when germ cells are absent (Coulombre and Russell, 1954).

In disagreement and in favor of the germ cells is Mintz (1969a) who examined fertile male XX ⟷ XY chimeras with functional germ line cells of XY when 95% of the somatic tissue of the gonad was made up of XX cells.

The role of both types of tissue are supported by the work of Milet et al. (1972) who examined four XX ⟷ XY chimeras and found no relationship between the phenotypic sex of the animal and the proportion of XY cells in skin, spleen, kidney, bone marrow, or cornea. They suggest that phenotypic sex is determined jointly by the somatic component and primordial germ cells but give no explanation of their hypothesis (McLaren, 1976).

In conclusion, the phenotypic determination of sex in allophenic mice is influenced by the sex of component strains, and the distribution of these components into the somatic and germ cells of the gonads.

C. Hair Pigmentation in Allophenic Mice

Coat color in allophenic mice offers a visible genetic marker for evaluating external chimerism, but has been found to be very unreliable for estimating the internal composition of the mice (Warner et al., 1976). Coat color is determined by the two cell types that make up the hair follicles of the animal. Coat color can be influenced by genes active in the melanocytes that determine the type and quantity of pigment in the hair shaft. It is also influenced by the genes of the epidermal cells that determines the pattern of how the hair is laid down (McLaren, 1976). In allophenic mice, chimerism can exist at the level of individual hairs (McLaren and Bowman, 1969).

Coat color chimeras are classified as melanocyte chimeras, and hair follicle chimeras, depending upon the appearance of the animal (McLaren, 1976). In the melanocyte chimeras, coat color patterns tend to distribute

themselves in broad transverse bands of color over the head, body and tail of the mice. At the mid-dorsal line there appears to be a sharp discontinuity, so that each side appears to be independently determined (Mintz, 1967).

Mintz (1967, 1970, 1971b) hypothesized that the most frequent standard pattern in melanocyte chimeras was three bands over the head, six on the side and eight on the tail. Based on these observations, she suggested that there must be 34 primordial melanoblasts in the neural crest, with each arising from a single progenitor cell that was responsible for each of the transverse bands. This orientation reflected the route of cellular migration from the neural crest, at 8-12 days of gestation. The mid-dorsal line of discontinuity was the result of clones of melanoblasts migrating before the neural crest had a change to fuse, thereby giving the appearance that each side is independently determined (McLaren 1976). West (1975) has verified the accuracy of this hypothesis by showing mathematically that 34 clones would be required to produce 17 bands on each side. However, McLaren and Bowman (1969) when examining the transverse bands characteristic of melanocyte chimeras, observed the most frequent banding pattern was one band over the head, five bands over the body, and one band over the tail. They concluded that the distribution of bands observed by Mintz (1967) was not the most frequent, but the maximum number of bands possible.

Hair follicle chimeras, like melanocyte chimeras, show transverse bands, with a mid-dorsal discontinuity, except the bands are of a much finer nature (Mintz, 1970, 1971b). Coat color in these animals is

influenced by the hair follicle itself either through modifying the activity of melanocytes or by altering the structure of the hair (McLaren, 1976). Mintz (1969b, 1970, 1971b) has postulated that in agouti ⟷ nonagouti mice where the hair follicles were predominantly of one component, the pattern of banding may directly reflect the clonal history of the tissue and that each narrow band represents a single hair follicle clone and correlates with the number of somites in the area. She further suggested that coat color in these mice is of mesodermal origin and not ectodermal since mesoderm is known to contribute to the hair follicle in the form of the dermal papilla (McLaren, 1976).

In contrast, McLaren (1976) has suggested that hair follicle bands do not represent localized clones of progenitor cells, but instead some developmental phenomenon superimposed on a systemic wave pattern which arises from the presence of two genetically distinct cell populations. This theory would explain why the agouti locus is responsive to positional effects which result in hair of identical genotype producing different phenotypes. The same genotype produces yellow hairs on the stomach, agouti hairs on the side, and black hairs in the middle of the back (McLaren, 1976).

The dynamic nature of pigment patterns was shown by Warner et al. (1976) when characterizing the coat color of 17 pigmented ⟷ nonpigmented C57BL/6 ⟷ (A x SJL)F_1 mice at two time points. They observed 5 out of 17 or 29% of the population showed significant change in their coat color composition. In 4 out of 5 mice, the shift was in the direction of the

C57BL/6 phenotype, while one mouse shifted to the (A x SJL)F_1 phenotype. Changes in the relative proportion of parental cells with time were termed chimeric drift (Stephens et al., 1977; Warner et al., 1977c,d). Although no attempt was made to quantitate bands, evidence presented was not consistent with the single clone theory of Mintz (1969b, 1970, 1971b) which suggests the possibility of a developmental and(or) environmental phenomenon being exerted on these mice.

D. Stability of Allophenic Mice

It was first suggested by Mintz and Palm (1969) that allophenic mice may not be completely stable chimeras but may show changes in their cellular composition with age. In an extensive study, they examined the erythrocyte H-2 type and serum gamma globulin allotype in 34 adult C3H(f) $\longleftrightarrow$ C57BL/6 mice. Of the 34 mice tested, 18 mice appeared to have only C3H(f) or C57BL/6 erythrocytes while 16 mice gave evidence of possessing erythrocytes of both strains, as detected by antibody dependent agglutinination and absorption tests. The presence of hybrid red blood cells in these chimeric mice was excluded based on previous direct examination of allophenic mice, using the T6 translocation marker and electrophoretically distinct forms of isocitrate dehydrogenase or malate dehydrogenase of which each parental strain showed a homozygous allelic variant genotype (Mintz and Palm, 1969). Red blood cells and serum allotype of 7S gamma globulins (subclass IgG2a) showed some concordance to the erythrocyte composition in all but one of the mice, with half of the mice showing a single red blood population and a serum allotype

corresponding to a single parental strain. The results also suggested that the C57BL/6 erythropoietic tissue might have a selective advantage over the C3H(f) strain and that time dependent shifts in the direction of only the C57BL/6 phenotype occurred. This advantage seemed greater with respect to the erythrocytes than the gamma globulin producing cells.

Wegmann and Gilman (1970) also examined a series of C3H/He ⟷ C57BL/6 mice using the hemoglobin beta-chain marker and allotype analysis of IgG2a gamma globulin. Of the nine mice judged chimeric from coat color analysis, all contained hemoglobin and gamma globulin of both parental types, with no evidence of a selective advantage of the C57BL/6 red blood cells. Results further indicated no time dependent changes in the direction of either parental strain, but instead concordance between red blood cell and white blood cell (i.e., gamma globulin producing cells) populations at all time points sampled.

West (1977) investigated the relative proportions of the two red blood cell populations of eight C3H/BiMcL ⟷ C57BL/McL mice and ten (C57BL/McL x C3H/BiMcL)F_1 ⟷ Recessive (Recessive is a multiple recessive stock previously described by McLaren and Bowman, 1969) by using the hemoglobin beta-chain marker and electrophoretically distinct forms of GPI of which each parental strain showed a homozygous allelic genotype. Analysis of a large number of tissues and organs from the eight C3H/BiMcL ⟷ C57BL/McL aggregation chimeras suggested that the C57BL/McL red blood cell population predominates, whereas in other tissue the proportions of the two genotypes are nearly equal. Similar results were

obtained with the ten (C57BL/McL x C3H/BiMcL)F_1 ⟷ Recessive chimeras. Further analysis of this data suggests that a temporal shift in the proportions of the two component red blood cell populations occurs in some adult chimeras and results eventually in an unbalanced phenotype. This has been claimed in the case of some spontaneous chimeric cattle twins (Stone et al., 1964), artificial sheep chimeras (Tucker et al., 1974), and other allophenic mice (Mintz and Palm, 1969). Although temporal shifts of this nature are difficult to explain, it has been suggested (Mintz and Palm, 1969; West, 1977) that time dependent changes in erythropoietic tissue may be the result of differential rates of mitotic activity that contributes to strain-dependent, tissue-specific selective pressures in allophenic mice. One of the major goals of the work reported in this thesis was the quantitative evaluation of time-dependent changes in the red blood cell and white blood cell populations of allophenic mice.

E. The Immune System of Allophenic Mice

Studies characterizing the immune system (bone marrow, spleen, thymus, Peyer's patches, lymph nodes, and circulating lymphocytes) of allophenic mice indicate that the lymphomyeloid system can be chimeric, but the ratio of the two parental strains in different tissues and organs is not uniform (McLaren, 1976). Gornish et al. (1972) examined five allophenic mice--three C3H/He ⟷ (C3H/He x CBA/H-T6)F_1 and two C57BL/10 ⟷ (C57BL/10 x CBA/H-T6)F_1 -- produced by the aggregation of two 8-cell embryos according to Mintz (1962a,b). From a karylogical analysis of the

mice, they concluded that the percentage of cells carrying the T6 chromosome marker was the same for bone marrow, spleen, and thymus. Their results have been taken as support for the view that lymphomyeloid tissue throughout the body arises from a single stem cell pool (McLaren, 1976).

In another study Ford et al. (1975) characterized two allophenic mice which were produced by the injection of three to five cells into the inner cell mass of recipient blastocysts according to the method of Gardner (1968). The mice were of the type PDE $\longrightarrow$ (PDE x CBA/H-T6)F_1 where the PDE is a random-bred albino strain. Their results indicated that the population of T6 containing cells was similar in bone marrow, thymus, and Peyer's patches, but different from the proportion of T6 containing cells in a second group of tissues, the spleen and lymph nodes. Attempts to explain the inconsistencies between these two sets of findings are difficult because of technical difficulties in both studies. In the Gornish et al. (1972) study, there appears there might have been a selective advantage of F_1 cells over C3H cells, thereby accounting for some of the uniformity seen in tissue analysis. This is particularly true in chimeras such as AKR $\longleftrightarrow$ CBA/H-T6T6 mice where coat color and germ line analysis of the component strains are more or less equal, while in the lymphomyeloid system, AKR appears to have a selective advantage (Tuffrey et al., 1973; McLaren, 1976). In the Ford et al. (1975) investigation, observations are complicated by the fact that blood was cultured in the presence of phytohemagglutinin (PHA) and the two genetic populations of lymphocytes could react differently to <u>in vitro</u> conditions or to stimulation with PHA.

Bona et al. (1974) analyzed the parental distribution of theta antigens in eight AKR ⟷ CBA/H-T6T6 chimeras using immunoautoradiography. When the two lymphocyte populations were distinguished by their theta antigens, the proportions of cell types were almost equal, ranging from 24% to 76% AKR; with a mean of 46%. In contrast to control F_1 animals, no cells in the chimeras carried both antigens implying that cell hybridization seldom if ever occurs. This latter observation is consistent with the studies of Mintz and Palm (1969).

In contrast, cell hybridization of lymphocytes in allophenic mice has been reported by other investigators (Munro et al., 1974a). In experiments designed to test if genetic information for immunoglobulin synthesis is transferred between cells as part of the normal *in vivo* response to antigen, allophenic mice of the combination BALB/c ($\underline{H\text{-}2}^d$, Ig^a) ⟶ C57BL ($\underline{H\text{-}2}^b$, Ig^b) were constructed between the two strains by transferring the inner cell masses of BALB/c blastocysts into C57BL blastocysts according to Gardner (1968). The surface antigens were detected by reacting suspensions of cells with appropriate anti H-2 serum followed by C6-deficient rabbit complement and fluorescently labeled sheep anti rabbit C3. After the treatment, viable cells were fluorescently stained and able to secrete immunoglobulins. Indirect plaque forming cells were detected by mixing cells with anti-IgM antiserum in agar and spreading the mixture on glass slides. The agar-covered slides were then treated with anti-allotype serum and complement, specific for one of the parental cell types in the population. By combining the indirect plaque assay with

immunofluorescence it was possible to estimate the number of cells of a haplotype making antibodies of the wrong allotype. In the majority of the nine chimeras analyzed, no apparent gene transfer occurred, but this was not true for two mice. No further attempts have been made to characterize other parental combinations of allophenic mice for hybrid cells.

F. Allophenic Mice as Test Systems for Cellular Cooperation Across H-2 Barriers

In recent years, the immune response of mice to synthetic polypeptide antigens, alloantigens and isoantigens and limiting doses of complex foreign proteins has been demonstrated to be under the control of a number of specific immune response (Ir) genes within the major histocompatibility (H-2) complex (Klein, 1975). However, the nature of the Ir gene products and their functions remain unknown. They may be expressed in T cells, B cells or macrophages and may be involved in cell-cell interactions among these cell types.

In attempting to answer these questions, investigators have constructed chimeric mice between two inbred strains, one of which was capable of producing antibody to a particular antigen and the other which was not. The antigens which have been used include (poly-L tyrosine, L glutamic acid)-poly D,L alanine--poly L lysine [(T,G)-A--L], (Freed et al., 1973; Bechtol et al., 1974; Bechtol and McDevitt, 1976; Press and McDevitt, 1977); 2,4-dinitrophenyl L-glutamic acid^{57}, L-lysine^{38}, L-alanine^{5} (DNP-GLA^{5}); (Warner et al., 1973); L-glutamic acid^{60}, L-alanine^{30},

L-tyrosine^{10} (GAT^{10}) (Warner et al., 1973) and poly(glutamic acid^{56}, lysine^{35}, phenylalamine^{9}) (GLØ) (Warner et al., 1976, 1977a).

Katz et al. (1973) first demonstrated a restriction in lymphycyte cell interaction to an antigen under immune response (Ir) gene control by using an adoptive transfer system. T cells from (low responder x high responder)F_1 mice previously primed to the terpolymer GLT can collaborate with (DNP)-primed B cells from the high responder strain but not a low responder strain in response to DNP-GLT. In other studies (von Boehmer et al., 1975b) with bone marrow reconstituted tetraparental mice have demonstrated that T cells can interact with H-2 histoincompatible B cells in response to antigens not under Ir gene controls.

In similar experiments by Sado and Kamisaku (1975) the antibody response to sheep red blood cells of syngeneic B cell-bearing radiation chimeras receiving syngeneic, semiallogeneic or allogeneic macrophages and T cells in various combinations were analyzed. Results indicated that histoincompatible T and B cells would not cooperate successfully for antibody production, while semiallogeneic T cells cooperated successfully with B cells in the chimeras.

Other investigators (Munro et al., 1974b; Munro and Taussig, 1975; Taussig et al., 1974, 1976; Mozes et al., 1975; Waldmann et al., 1975) characterizing the immune response to the synthetic polypetides (T,G)-A--L, (T,G)-P--L, and (Phe,G)-A--L have shown that specific soluble T cell factors from in vitro culturing of thymocytes can replace T cells in vitro and can cooperate with H-2 histoincompatible B cells in producing direct plaques. Their results indicate that defects in responsiveness to an

antigen can be found at the level of the B cell (lacks genes coding for an acceptor for a class of T cell factors), T cell (lacks a gene coding for a specific binding site for antigen) or in both cell types depending upon the strain of mice used.

The *in vivo* response of tetraparental mice immunized with (T,G)-A--L has been investigated by Bechtol et al. (1974) and Bechtol and McDevitt (1976). In their studies several tetraparental mice produced anti-(T,G)-A--L antibody of the low responder strain gamma globulin allotype. Their data implied that the *Ir-IA* gene controlling the response to (T,G)-A--L was not expressed in B cells and suggested that cellular interactions among genetically dissimilar cell populations could occur when tolerance existed to both cell types. Attempts to clarify these observations by using bone marrow cell chimeric mice (presumed tolerant to *H-2* histoincompatible cells) to examine antigen specific and allotype specific antibody suggests that low responder B cells cannot interact with chimeric histoincompatible high responder T cells and macrophages in an antigen under *Ir* gene control (Press and McDevitt, 1977). Thus, the bone marrow and allophenic mouse data on the (T,G)-A--L system are conflicting. However, Press and McDevitt (1977) now claim that attempts to repeat the original observations on the allophenic mouse sera have given no evidence of antibody of the low responder allotype.

Warner et al. (1978) when examining the antibody response of 13 C57BL/6 $\longleftrightarrow$ A mice immunized to $DNP_{56}BGG$ confirmed these results. They observed that none of the mice, in spite of the presence of a significant

proportion of low responder cells in some mice, produced any significant antibody of the low responder allotype. Their conclusion was that two histoincompatible cell types in allophenic mice, one from a high responder strain and one from a low responder strain to $DNP_{56}BGG$ is not sufficient to allow the low responder cells to produce anti-DNP antibody.

G. Transplantation and Immunological Tolerance

1. The H-2 complex

Transplantation or grafting is defined as the transfer of living cells, tissues, or organs from one part of the body to another, or from one individual to another (Klein, 1975). The success of the graft is primarily determined by the genetic relationship between the donor and the host. The immunological basis of rejection of normal tissue was first demonstrated by Medawar and his colleagues (Billingham et al., 1954). In general, their investigations indicated that grafts (isografts) between genetically identical individuals (syngeneic) would succeed, while grafts (allografts) between genetically different individuals (allogeneic) would not succeed. Rejection was typically characterized by dilation of the blood vessels with erythrocytes, followed by hemorrhages and the invasion of a mixed population of lymphoid cells into the grafted area. This inflammatory reaction spread upward through the dermis and epidermis until edema, necrosis, and tissue breakdown ultimately resulted in a sloughing off of the graft (Klein, 1975). The complex physiological phenomenon associated with graft rejection in the unsensitized individual

is referred to as a first set reaction, while the response of a sensitized individual to a graft is referred to as a second set reaction (Klein, 1975). The success of transplantation can be summarized into five laws (Snell and Stimpfling, 1966; Klein, 1975);

1. Grafts within an inbred strain (syngeneic grafts) succeed.

2. Grafts between different inbred strains (allografts) fail.

3. Grafts from either inbred parent strain to the F_1 hybrid succeed, but grafts in the reverse direction fail.

4. Grafts from F_2 or subsequent F generations to F_1 succeed.

5. Grafts from either inbred parent strain succeed in some members of an F_2 generation but fail in others. Also, grafts from one inbred parental strain succeed in some members and fail in others of a backcross produced by crossing the F_1 hybrid to the opposite parental strain.

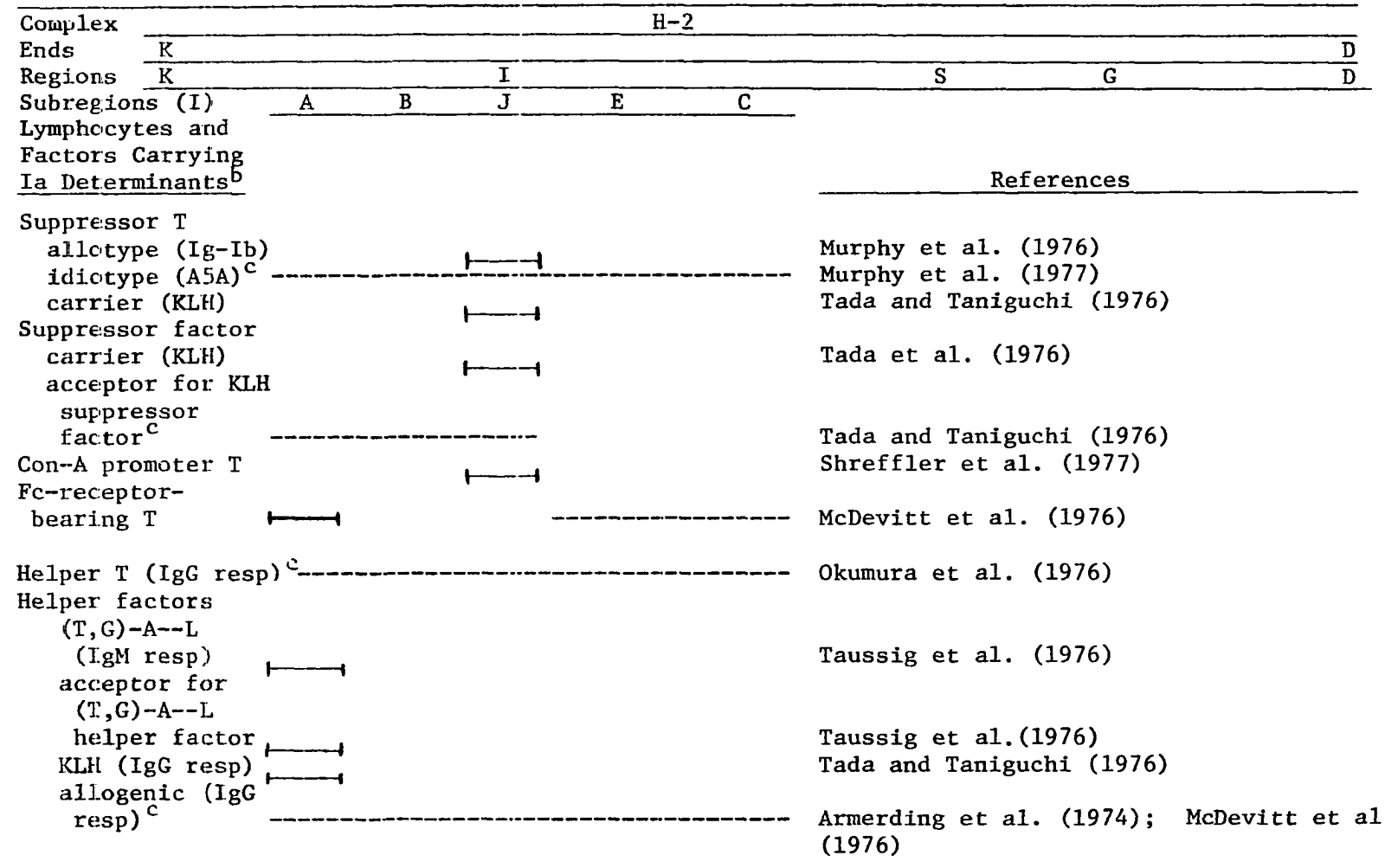
The factors responsible for tissue compatibility were previously identified by Snell et al. (1948, 1953) as histocompatibility antigens and the genes coding for these structures as histocompatibility genes. In the mouse, the histocompatibility genes involved in governing transplantation and graft rejection are called the H-2 complex and can be mapped to a segment of chromosome 17 (Klein, 1975). The particular combinations of H-2 antigens coded by this segment of chromosome 17 are called the H-2 haplotype and is typically designated by small letters in the superscript position (e.g., $H\text{-}2^a$)(Klein, 1975).

However, only in recent years have investigators appreciated the serological and genetic complexity of the H-2 complex and its antigens.

The current working model is that the antigens are encoded by two loci situated at either end of the major histocompatibility complex, the H-2K and H-2D regions (Snell et al., 1971) (see Table 1 for a map of the H-2 complex). The I region was originally defined by the immune response genes controlling the response to thymus-dependent antigens (McDevitt et al., 1972) discussed in the previous section. However, it has recently been found that the antigens in this area stimulate a variety of cell mediated responses such as mixed lymphocyte culture (MLC) and graft-versus-host response (GVH)(Klein, 1975; Brent et al, 1976). Also immune functions such as the generation of cytotoxic T cells in vitro not only depend upon differences between responder and stimulator cells in the H-2K and H-2D ends, but also in the I region (Cantor and Boyse, 1975a,b). The cytotoxic cells themselves are directed against the H-2K and H-2D ends but for optimal response they require the help of cells that recognize I region antigens and proliferate in response to them (Schendel and Bach, 1974; Cantor and Boyse, 1975a,b; Brent et al., 1976). The cells generated by immune functions can often be separated based upon different surface antigens such as the Ly differentiation antigens (Cantor and Boyse, 1975a,b) and the Ia antigens (Shreffler and David, 1975).

The I regions consist of five subregions designated IA, IB, IJ, IE, and IC. I subregion antigens are selectively expressed on helper and suppressor T cells and play a role in the regulation of humoral responses. A new subregion, IJ has been found to control a determinant found on allotype suppressor and normal T lymphocytes (Murphy et al., 1976).

Table 1. Genetic nomenclature of the H-2 complex[a]



B lymphocytes	⊢——⊣	⊢————————⊣	Sachs and Cone (1973); Hämmerling et al. (1974); Götze (1975); David et al. (1975); Shreffler et al. (1977)

[a]From Klein (1975); Murphy et al. (1977).

[b]I subregions localization of loci controlling determinants present on different lymphocyte subpopulations and immune factors.

[c]Not mapped to a subregion.

The S region, located between the I region and the G region contains two loci which code for serum proteins (Shreffler and Owen, 1963). The Ss locus controls the levels of serum complement, particularly C4, while Slp locus controls the expression of a sex limited testosterone dependent allotypic marker present on the serum substance (Ss) (Shreffler and Passmore, 1970). This dominant trait is expressed on some, but not all, Ss proteins (Klein, 1975). The G region contains the H-2G locus which controls erythrocyte antigens (Shreffler and David, 1975). The K and D regions are considered peripheral regions of the H-2 complex while the I, S and G regions are central regions (Klein, 1975).

2. Tolerance

Under certain conditions, the transplant recipient may be rendered unresponsive to foreign grafts. The state of unresponsiveness is called immunological tolerance (Klein, 1975). In self-tolerance, all reactive lymphoid cells to self-components are passively eliminated or actively suppressed.

a. The forbidden clone theory Although several theories have postulated the mechanism of self-tolerance, only one has accurately accounted for both immune responsiveness and tolerance. The forbidden clone theory (Burnet, 1959; Jerne, 1971) postulates that self-tolerance is induced by specific deletion or irreversible inactivation of potentially reactive clones to self-antigens during ontogeny. This is based on the premise that each lymphoid cell carries one immunological pattern expressed as a receptor which is passed on to its clonal descendants.

Clonal deletion of cells carrying receptors for self are absent for as long as the antigen which induces deletion is present. However, removal of the tolerizing antigen may then allow the generation of new precursor cells from noncommitted stem cells. This observation is supported by numerous reports of the absence of specific GVH and MLC reactive cells in mice and rats made tolerant by the neonatal injection of a large number of foreign cells (Gowens et al., 1963; Schwarz, 1968; Atkins and Ford, 1972; Brent et al., 1972; Brooks, 1975; Brent et al., 1976). When suboptimal dosages of foreign cells were given to recipients, residual T cells are thought to be present and give rise to low but detectable GVH and MLC reactions (Gowens et al., 1963; Bernstein, 1975; Brooks, 1975). Evidence has shown that tolerance in these neonatal chimeras was not caused by the active suppression of host T cell reactivity (von Boehmer and Sprent, 1976). Autoimmunity according to the clonal selection theory is thought to result from either the somatic mutation and proliferation of lymphocytes into self-reacting clones; changes in the nature of the autoantigen perhaps due to viral infection; exposure to antigens hidden from the immune system during ontogeny; immunization to antigens cross reactive to autoantigens or from intense stimulation of lymphocyte proliferation (Talal et al., 1976).

Zinkernagel and Doherty (1974a,b,c) have shown for T cell mediated cytotoxicity reactions that T lymphocytes from lymphocytic choriomeningitis (LCM)-infected mice could lyse LCM-infected targets only if they expressed the same H-2 haplotype as the donor of the effector cells. In

their model, they hypothesize the viral immune effector T cells express two variable region (V) genes, one specific for self and the other for virus-induced changes. Virus-induced cytotoxic T cells generated in a mouse which is heterozygous at the H-2 complex would, therefore, express one of four self-reactive V genes specific for each of the alleles at H-2K or H-2D. In self-tolerance, clones that express two V genes specific for self would be deleted or suppressed whereas those which possess recognition structures for self or nonself (surveillance T cells) or two components for nonself (alloreactive T cells) will enter the recirculating pool. Autoimmunity or altered self is the result of changes in H-2 antigens induced by the process of virus synthesis or as some complex of viral and H-2 antigens. This suggests that both the infecting or modifying agent is in close association with the self H-2 products, and are recognized by the cytotoxic T lymphocytes as a single receptor (Zinkernagel 1976a,b,c; Zinkernagel and Doherty, 1974a,b,c, 1975, 1976; Zinkernagel and Welsh, 1976). However, an altered self model and a dual recognition model cannot be distinguished yet. Similar results have been obtained with a chemically modified cell mediated lympholysis model (Shearer and Schmitt-Verhulst, 1977). Cells modified by TNP separated from the cell surface by a tripeptide did not act as targets for effectors generated by sensitization against TNP-modified cells (Rehn et al., 1976). If T cell recognition occurs by two independent receptors, one recognizing the TNP group and the other recognizing self H-2 coded products, either or both of the targets modified by these slight variations in TNP presentation on the cell surface should have been recognized by

effector cells specific for TNP-self. These findings are compatible with recognition of single or double receptors and an altered self model or dual recognition model (Shearer and Schmitt-Verhulst, 1977).

However, the H-2 restriction requirement for viral infected T cell mediated cytotoxic reactions has recently been reexamined (Zinkernagel et al., 1978). Using lethally irradiated bone marrow reconstituted chimeras ($F_1 \longrightarrow$ parent), they found tolerance alone was not sufficient to generate both virus specific and TNP-specific cytotoxic T cells against antigen in association with the tolerated H-2 types. In their experiments adult thymectomized irradiated C57BL/6 were reconstituted with bone marrow of (BALB/c x C57BL/6)F_1 origin and transplanted along with two irradiated thymus lobes of the F_1 hybrid. The thymus lobes were transplanted under the kidney capsule, immediately following bone marrow reconstitution. The reconstituted mice were infected with vaccinia virus. After 5-10 days, F_1 hybrid cytotoxic T cells were formed against virus infected parental target cells of either H-2 type. In contrast, control nonthymectomized irradiated bone marrow chimeras ($F_1 \longrightarrow$ parent), reconstituted with bone marrow of F_1 origin but not receiving a F_1 thymus prior to infection with vaccinia virus, formed cytotoxic T cells whose specificity of killing was limited to virus infected target cells of the same H-2 haplotype as the parental recipient component of the reconstituted mice.

There are a number of conclusions that can be drawn from these experiments. First, it appears the thymus plays an important role in the differentiation of H-2 restricted killer T cells. Their capacity

for self-recognition or recognition of modified target cells is determined primarily by the radioinsensitive portion of the thymus, the thymus epithelium. Second, these findings are more compatible with a dual recognition model, where the antigen specific cytotoxic T cells recognize two receptors on target cells. The two receptors would consist of a virally modified H-2 determinant and a second separate receptor whose recognition by educated killer T cells is determined during the course of their maturation in the thymus. The biochemical and serological nature of this latter receptor presently remains unknown. Finally, there are some data which support a dual recognition model and other data which support an altered-self model. The controversy is unresolved.

b. Other theories of tolerance In contrast to the forbidden clone theory, other investigators have hypothesized that neonatally induced tolerance is mediated through the action of serum blocking factors (Voisin et al., 1972; Hellström et al., 1970, 1971; Wegmann et al., 1971; Phillips et al., 1971; Bansal et al., 1973a,b). This theory is based upon numerous reports that self-reactive clones of lymphocytes are retained in fully tolerant mice and rats (Hellström et al., 1971; Wegmann et al., 1971; Phillips et al., 1971; Phillips and Wegmann, 1973; Bansal et al., 1973a; Wright et al., 1974a,b; and Festenstein et al., 1975). The blocking factor is thought to be either an antibody or an antibody-antigen complex that might be directed specifically against the relevant receptor sites on the surface of immunologically competent cells (McLaren, 1976). The presence of serum blocking activity has been reported

in allograft tolerant chickens containing cells with GVH reactivity against the tolerant strain (Droege and Mayor, 1975). Contrary to these observations, other studies have shown tolerant serum to have no effect on either GVH or MLC reactivity of normal cells (Ceppellini, 1971; Atkins and Ford, 1972; Brent et al., 1972; Crone, 1973; Meo et al., 1973; Festenstein et al., 1975). It has also been demonstrated that tolerance can be induced in agammaglobulinemic chickens just as easily as in normal chickens (Rouse and Warner, 1972; Crone, 1973; Nowygrod et al., 1974).

Another theory of tolerance is based on the observation that T lymphocyte receptors and IgG molecules share similar idiotypic determinants (Binz and Wigzell, 1975; Brent et al., 1976). Anti-T cell receptor antibodies can be raised to the variable portion because of the idiotypic determinants which are in effect a protein new to the organism; with its determinant group as an antigen being its idiotype. Anti-recognition structure (anti-RS) can be raised by injecting semi-allogeneic cells into recipients of one or the other parental strains, with donor cells forming anti-RS against host lymphocyte receptors for alloantigens of the opposite parental strain (Ramseier, 1973a,b; Binz and Wigzell, 1975). Such anti-RS antibodies have been shown to selectively destroy the ability of recipient strain lymphocytes to mount GVH reactions (Binz, 1975) and to inhibit cell mediated cytotoxicity and the mixed lymphocyte reaction (Kimura, 1974; Binz and Askonas, 1975). However, no direct evidence has shown that anti-RS antibodies play a role in neonatally induced tolerance.

Suppressor cells have recently been described as mediating neonatal self-tolerance. One of the best examples of T cell suppressor cells is the experiments of Pinto et al. (1974). In their experiments recipient mice given an i.v. injection of donor strain liver extract, followed by an i.v. injection of B pertussis were grafted and treated with anti-lymphocyte serum for three days. Recipient mice were found to maintain healthy grafts for months after transplantation while maintaining a reactive population of lymphoid cells to donor antigens as determined by GVH, MLC and cell mediated lympholysis assays (CML) (Brooks et al., 1975). This tolerance can be passively transferred to syngeneic donor mice pretreated with sublethal irradiation or antilymphocyte serum after transfer. Pretreatment of the population of cells with anti-theta serum and complement before transfer, abolishes the effect (Kilshaw et al, 1975; Brent et al., 1976). Recently it has been shown that recirculatory suppressor T cells play an important role in transplantation tolerance in rats (Dorsch and Roser, 1977).

In chickens pretreated with a sublethal dosage of irradiation, prior injection of syngeneic thymus cells prolonged the survival of skin allografts (Droege, 1975). This population of suppressor cells have been characterized as being found predominantly in the thymus of young chicks and are bursa dependent. These thymus cells after exposure to the antigen can lead to the formation of specific suppressor cells that can mediate long term tolerance to that antigen (Droege, 1976). An earlier study by Rouse and Warner (1974) has shown that chickens made tolerant in early

embryonic life possess suppressor cell activity, but no attempt was made in the identification of the effector population. In contrast, several other groups of workers have shown that lymphoid cells from tolerant rats and mice do not suppress normal syngeneic lymphocytes (Atkins and Ford, 1972; Meo et al., 1973; Brooks, 1975; Festenstein et al., 1975).

H. Tolerance in Allophenic Mice

Allophenic mice provide an unique model for the study of immunological tolerance. We have seen that allophenic mice, although chimeric for lymphoid cell populations, appear mutually tolerant to the other component strain cells. Although a number of experiments have been performed trying to elucidate the mechanism of this tolerance, there is no consensus of opinion at the present time on the true mechanism. Similarly, very little is known about the cellular composition of the immune system of allophenic mice. Previous studies characterizing the immune system of allophenic mice have been plagued by a number of difficulties. Among the problems commonly seen is the application of qualitative techniques for quantitative data collection, the use of small numbers of mice with no parental strain combination diversity, and the employment of techniques leading to biased estimation of the relative proportions of the two cell types in a tissue or organ. In spite of the lack of understanding of the immune system of allophenic mice, these mice have been and currently are being used in a number of important immunological studies (McLaren, 1976).

The work described in this dissertation was undertaken to characterize and quantitate the hematopoietic and immune systems of a large, diverse population of allophenic mice. Techniques were developed to examine erythrocyte, peripheral white blood cell, spleen white blood cell and thymocyte compositions of the mice. This approach was accomplished by using polyacrylamide gel isoelectric focusing (PAGIF) and antibody mediated cytotoxicity. Mice were analyzed for changes in their peripheral white blood cell composition and hemoglobin composition as a function of time. Spleen white blood cell and thymocyte compositions were analyzed at the time of sacrifice. These studies were carried out concomitantly with a project that was aimed at evaluating the possible role of serum blocking factors as a mechanism of tolerance in allophenic mice.

II. EXPERIMENTAL PROCEDURES

A. Experimental Animals

1. Mice

All inbred strains of mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The strains used in these studies were C57BL/6, C57BL/10Sn, CBA, CBA/H-T6, DBA/1, and SJL. The F_1 hybrids (CBA x CBA/H-T6) and (A x SJL) were bred in our laboratory. The choice of inbred strains and F_1 hybrids was based on genetic differences at the major histocompatibility (H-2) complex, and at the gene for the β chain of hemoglobin. This information is summarized in Table 2 . Inbred mice of the same sex were kept in metal cages in groups of eight and were given mouse chow and water ad libitum. The room was kept light from 7 a.m. to 9 p.m. and dark from 9 p.m. to 7 a.m.

2. Allophenic mice

Allophenic mice used for these studies were constructed in our laboratory by J. McIvor, C. Tollefson, and C. Warner. The allophenic mice were produced by the techniques of Tarkowski (1961) and Mintz (1962a,b, 1971b) as modified by Warner et al. (1973). Mature female mice were superovulated by an intraperitoneal injection of 5-10 I.U. of pregnant mare serum (Organon)(PMS), followed 48 hours later by 5-10 I.U. of human chorionic gonadotropin (Nutritional Biochemical, Cleveland, Ohio) (hCG). Both injections were at about 4 p.m. Females were then mated with males

Table 2. Description of inbred, F_1 hybrid and allophenic mice[a]

Mouse Strain	Hemoglobin[b] Type	H-2 Haplotype	Tla Haplotype
A. Inbred Strains			
C57BL/6	single	b	b
C57BL/10Sn	single	b	b
DBA/1	diffuse (double)	q	b
CBA	diffuse (double)	k	b
CBA/H-T6	diffuse (double)	k	b
A	diffuse (double)	a	a
SJL	single	s	a
B. F_1 Hybrids			
(CBA x CBA/H-T6)F_1	diffuse (double)	k	b
(A x SJL)F_1	diffuse (double)-single	as	a

[a]Based on Klein (1975).

[b]This refers to the genetic polymorphism in Hbb (Gilman, 1972; Hutton et al., 1962a,b). Electrophoresis shows Hbb either as a "single" or as a "diffuse" species. In our study, isoelectric focusing further defines the diffuse Hbb as two bands, which we have referred to as "double" hemoglobin.

[c]• = Unknown.

[d]— = absence of antigen.

Thy-1	Ly 1	Ly 2	Ly 3	Ly 4	Ly 5
2	2	2	2	2	1
2	2	2	2	2[d]	1
•[c]	1	1	2	—	•
2	1	1	•	•	•
2	1	1	•	•	•
2	2	2	2	—	1
•	2	2	2	—	2
2	1	1	•	•	•
2/•	2	2	2	—	1/2

Table 2. (continued)

Mouse Strain	Hemoglobin[b] Type	Haplotype H-2	Haplotype Tla
C. Allophenic Mice			
C57BL/6 ↔ (A x SJL)F_1	single ↔ diffuse (double)	b ↔ as	b ↔ a
DBA/1 ↔ (A x SJL)F_1	diffuse (double) ↔ diffuse (double)	q ↔ as	b ↔ a
DBA/1 ↔ A	diffuse (double) ↔ diffuse (double)	q ↔ a	b ↔ a
(CBA x CBA/H-T6)F_1 ↔ DBA/1	diffuse (double) ↔ diffuse (double)	k ↔ q	b ↔ b
(CBA x CBA/H-T6)F_1 ↔ C57BL/6	diffuse (double) ↔ single	k ↔ b	b ↔ b
(CBA x CBA/H-T6)F_1 ↔ A	diffuse (double) ↔ diffuse (double)	k ↔ a	b ↔ a
C57BL/6 ↔ DBA/1	single ↔ diffuse (double)	b ↔ q	b ↔ b
C57BL/6 ↔ A	single ↔ diffuse (double)	b ↔ a	b ↔ a
C57BL/10Sn ↔ A	single ↔ diffuse (double)	b ↔ a	b ↔ a

Haplotype					
Thy-1	Ly 1	Ly 2	Ly 3	Ly 4	Ly 5
2 ↔ 2/•	2 ↔ 2	2 ↔ 2	2 ↔ 2	2 ↔ —	1 ↔ 1/2
• ↔ 2/•	1 ↔ 2	1 ↔ 2	2 ↔ 2	— ↔ —	• ↔ 1/2
• ↔ 2	1 ↔ 2	1 ↔ 2	2 ↔ 2	— ↔ —	• ↔ 1
2 ↔ •	1 ↔ 1	1 ↔ 1	• ↔ 2	• ↔ —	• ↔ •
2 ↔ 2	1 ↔ 2	1 ↔ 2	• ↔ 2	• ↔ 2	• ↔ 1
2 ↔ 2	1 ↔ 2	1 ↔ 2	• ↔ 2	• ↔ —	• ↔ 1
2 ↔ •	2 ↔ 1	2 ↔ 1	2 ↔ 2	2 ↔ —	1 ↔ •
2 ↔ 2	2 ↔ 2	2 ↔ 2	2 ↔ 2	2 ↔ —	1 ↔ 1
2 ↔ 2	2 ↔ 2	2 ↔ 2	2 ↔ 2	2 ↔ —	1 ↔ 1

and checked for vaginal plugs the next morning. Collection of 8-cell embryos from plug-positive mice was begun 64 hours after hCG administration. The 8-cell embryos were flushed from the oviducts using Whitten and Biggers (1968) medium. The zona pellucida was removed with the enzyme pronase and the embryos were mechanically bumped together. The embryos were allowed to culture for 24-36 hours at 37° C in an atmosphere of 5% CO_2 in air. The blastocysts were then transferred to a pseudopregnant CF1 female (Charles River, Portage, Michigan), previously mated to a vasectomized male. Mice were allowed to come to term.

The choice of inbred strains for allophenic mouse production was based on the hemoglobin and major histocompatibility (H-2) type of the parental strains comprising an allophenic mouse. The allophenic mice used for these studies and their pertinent genetic markers are listed in Table 2.

B. Buffers and Culture Media

The various buffers and culture media used can be found in Table 3. All solutions were routinely prepared every 7-10 days.

C. Sera

1. Normal sera

Normal sera from inbred strains and allophenic mice were obtained by bleedings from the orbital venus sinus. Approximately 0.30 ml of whole blood was collected (Beckman Microfuge; 0.4 ml tube) and samples were permitted to stand at room temperature until the sera began to separate from

Table 3. Buffers and culture media

A. Isoelectric Focusing Buffers

1. 2X Hemoglobin Reducing Buffer pH 8.7

0.2M boric acid	12.40g
0.08M NAOH	3.20g
0.01M EDTA	2.92g
0.02M 2-mercaptoethanol	1.08ml
distilled H_2O to	1000ml

2. Sucrose-Cyanide Diluting Buffer pH 8.7

0.1M boric acid	6.20g
0.04M NAOH	1.60g
0.005M EDTA	1.46g
0.04M KCN	2.60g
sucrose	50g
distilled H_2O to	1000ml

3. Electrolytes

Catholyte		Anolyte	
0.02M H_3PO_4	1.96g	0.01M NAOH	0.4g
distilled H_2O to	1000ml	distilled H_2O to	1000ml

4. 10% TCA Solution (w/v)

trichloroacetic acid	100g
distilled H_2O	1000ml

B. Cytotoxicity Assay Solutions

1. RPMI 1640 with F.C.S.

RPMI 1640 with 25 mM Hepes buffer	90ml
heat inactivated fetal calf serum	10ml
millipore	.45 μm filter

Table 3. (continued)

2. L-15 with F.C.S.

L-15 culture medium	90ml
heat inactivated fetal calf serum	10ml
millipore	.45 μm filter

3. Saline pH 7.0

0.15M NaCL		8.50g
distilled H_2O	to	1000ml

4. EDTA Saline pH 7.0

0.001M EDTA		.29g
0.15M NaCl		8.50g
distilled H_2O	to	1000ml

5. Phosphate buffered saline (PBS) pH 7.0

0.003M $NaH_2PO_4 \cdot H_2O$		0.45g
0.007M $Na_2HPO_4 \cdot 12H_2O$		2.40g
0.13M NaCl		7.40g
distilled H_2O	to	1000ml

C. RNA Synthesis Assay Buffers

1. Supplemented RPMI 1640

RPMI 1640 with 25mM Hepes buffer	90ml
penicillin	1000 IU
streptomycin	100 μg
200mM L-glutamine	1.0ml
heat inactivated fetal calf serum	10ml
millipore	.45 μm filter

2. Carrier RNA (CRNA)

yeast ribonucleic acid	0.80g
distilled H_2O	100ml
heat to 40°C and filter	

Table 3. (continued)

3. Deoxycholate Solution

deoxycholate	12g
distilled H_2O	100ml
heat to 40-45° C	

4. Tris-BSA Washing Solution

0.01M Tris	Tris - HCl	0.33g
	Tris Base	0.049g
0.5% bovine serum albumin		1.25g
distilled H_2O	to	1000ml

5. Sodium Phosphate pH 9.2

0.5M Na_2HPO_4 (anhydrous)		70.98g
distilled H_2O	to	1000ml

D. Cell Freeze-Thawing Solution

1. 14% DMSO pH 7.0 (w/v)

Phosphate buffered saline PBS pH 7.0	88ml
14% dimethylsulfoxide (p = 1.096)	12ml

2. Cell Washing Solution

RPMI 1640 with 25mM Hepes buffer	50ml
penicillin	1000 IU
streptomycin	100 μg
200mM L-glutamine	1.0ml
heat inactivated human AB normal serum	50ml

the clot. The blood samples were then placed in the refrigerator for 5-10 hours. Blood clots were removed from sera with a disposable applicator stick and samples were centrifuged for 5 minutes in a Beckman Microfuge B (Beckman Instruments, Chicago, Illinois). Samples were pooled, if appropriate, heat inactivated, coded, and stored at a -70° C until needed.

2. Preparation of antisera

Antisera were produced against whole spleen cells according to the method of Batchelor (1973). Single cell suspensions were prepared by flushing spleens with 1.0 ml RPMI 1640 medium (Gibco, Grand Island, New York, or ISI Biologicals, Cary, Illinois) followed by pressing through a 40 or 50 mesh stainless steel screen. Each recipient mouse received 20-40 mg wet weight of the spleen cell suspension in 0.2 ml of RPMI 1640. This usually consisted of two donor spleens per eight recipient mice. Females were always used as donors and either males or females as recipients. Therefore, no anti-HY antibody was produced. Weekly injections (usually at least 10) were given until high titer antisera were produced. The recipient mice were alternately bled and boosted and finally exsanguinated. The serum from each bleeding was pooled, heat inactivated and stored either at -20° C or -70° C. Reciprocal immunizations were performed to eliminate cross-reacting antibodies. For example, to produce antisera for usage on C57BL/6 $\longleftrightarrow$ A mice, C57BL/6 mice were injected with A spleen cells and A mice were injected with C57BL/6 spleen cells.

Antisera to thymocytes were produced by performing similar reciprocal immunizations with thymocytes from each of the strains used in the

production of the allophenic mice. All antisera were appropriately coded and stored in various-sized aliquots at a -70° C.

3. Commercial antisera

Specific anti-H-2 sera prepared in congenic lines were obtained from the Research Resources Branch, National Institutes of Health. Single vials containing the lyophilized antiserum were reconstituted with 1.0 ml deionized distilled water and stored in aliquots of 100 µl at -70° C. The sera used included anti-$\underline{K}^{k}\underline{D}^{k}$(D-2A-T), anti-$\underline{K}^{s}\underline{D}^{s}$(D-36A-R) and anti-$\underline{K}^{k}\underline{D}^{k}$ (D-3bA-Q).

4. Guinea pig serum

Normal guinea pig serum obtained from Miles Laboratory (Elkhart, Indiana) was used as a source of complement in antibody mediated cytotoxicity assays. Lyophilized serum was reconstituted with 25 ml of supplied diluent (sterile distilled water) and aliquoted and stored in 200 and 500 µl amounts at -70° C.

Occasionally, to remove natural antibodies to murine spleen cells and thymocytes, the guinea pig serum was absorbed with agarose using the modified procedure of Cohen and Schlesinger (1970). Briefly, 30 mg of dry agarose were added to 1.0 ml of reconstituted guinea pig serum (Beckman Microfuge; 1.5 ml tube) on ice. Samples were gently vortexed every 3 minutes. Absorption was complete within 10 minutes (Cohen and Schlesinger, 1970). Supernatants were then collected by a 5-minute centrifugation and the absorbed serum aliquoted.

D. Antibody Mediated Cytotoxicity Testing

1. Cell preparation

The antisera produced by immunization with spleen cells were tested against both peripheral white blood cells and spleen white blood cells. The antisera produced against thymocytes were tested against thymocytes. All antisera were evaluated using the trypan blue dye exclusion assay (Warner et al., 1976) or the RNA synthesis assay (Stephens and Warner, 1978). Cells were isolated from the thymus or spleen by injecting 1.0 ml of either L-15 medium (Gibco) containing 10% heat inactivated fetal calf serum or RPMI 1640 with 25 mM hepes buffer (ISI Biologicals or Gibco) into their respective capsules and forcing the thymic or splenic contents through a 40 or 50 mesh stainless steel screen. Single cell suspensions were prepared by passing clumps through a 1.0 ml sterile plastic tuberculin syringe equipped with a 26-gauge needle or by repeated pipetting with a Pasteur pipet.

Peripheral white blood cells were obtained by bleeding mice from the orbital venous sinus using four 75 μl heparinized capillary tubes (Sherwood Medical Industries, St. Louis, Missouri). Approximately 0.30 ml of collected whole blood were rapidly mixed with 0.70 ml of a 0.001M EDTA saline solution (pH 7.0)(See Table 3).

All three cell types were prepared for cytotoxicity testing by the Ficoll-Hypaque density gradient centrifugation method (Böyum, 1968). The method of preparation of Ficoll-Hypaque solution is shown in Table 4.

Table 4. Ficoll-Hypaque density gradient solution

A.

9% (w/v) Ficoll (Pharmacia, Piscataway, N.J.)--dissolve 9 grams Ficoll powder in 100 ml distilled water.

34% (w/v) Hypaque (Winthrop Laboratory, N. Y.)--dissolve 34 grams Hypaque powder into 100 ml distilled water.

B.

Specific Gravity of F-H (25° C)	Parts 9% Ficoll	Parts 34% Hypaque
1.076	65.3	34.7
1.078	64	36
1.080	62.7	37.3
1.084	60	40
1.090	56	54

C.

Check specific gravity with a hydrometer.

Aliquot and store in the freezer.

Depending on the source of the white blood cells, the density of the Ficoll-Hypaque used varied. Optimal separation of spleen cells and thymocytes was achieved using a density equal to 1.080, while best results for peripheral white blood cells were obtained with a density of 1.084. Roughly, one part cell suspension was layered over two parts Ficoll-Hypaque solution in either 0.4 ml or 1.5 ml polyethylene microfuge tubes. Tubes were centrifuged for three minutes in a Beckman Microfuge B (Beckman Instruments, Chicago, Illinois). Cells were then washed with tissue culture medium (spleen cells and thymocytes) or with 0.001M EDTA saline solution (PWBC), and centrifuged for five seconds. The cells (1-6 x 10^6 cells/ml) were resuspended in L-15 with F.C.S. or RPMI 1640 with F.C.S. (see Table 3).

In experiments evaluating RNA synthesis as a probe of antibody mediated cytotoxicity, unfractionated single spleen cell suspensions containing both red blood cells and white blood cells were prepared as above, by passage through a stainless steel screen and the cells were washed once, counted, and resuspended in RPMI 1640 with 25mM hepes buffer (ISI Biological or Gibco) supplemented with heat inactivated 10% fetal calf serum, 1000 IU/ml penicillin, 100 µg/ml streptomycin (Sigma Chemical Company, St. Louis, Missouri) and 1% of 200 mM glutamine (Gibco Company)(see Table 3). The final white blood cell concentration was adjusted to 6.0 x 10^6 cells/ml. Each assay tube received 20 µl of this suspension to give 12 x 10^4 spleen white blood cells per assay.

2. Trypan blue dye exclusion assay

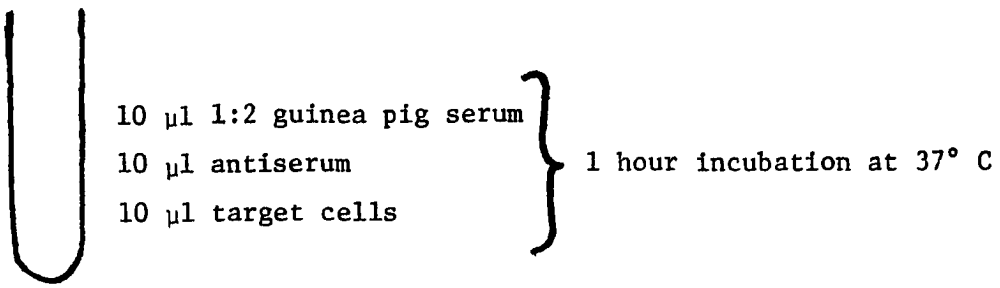
Two types of protocols were employed in the trypan blue dye exclusion assay, with selection depending upon the purpose of the experiment. These are summarized in Figure 2.

In routine histocompatibility typing, 10 µl of the Ficoll-Hypaque isolated cell suspension (6 x 10^6 cells/ml) were mixed with 10 µl of the appropriate antiserum dilution. Then 10 µl of a 1:2 dilution of guinea pig serum (Miles Laboratory) were added as the source of complement. The mixture was allowed to incubate at 37° C for one hour after which time 10 µl of a 0.4% filtered trypan blue solution was added (Gorer and O'Gorman, 1956).

A modified version of the protocol previously described was designed to compare the sensitivity and accuracy of the trypan blue dye exclusion assay with the RNA synthesis assay (described below)(Stephens and Warner, 1978).

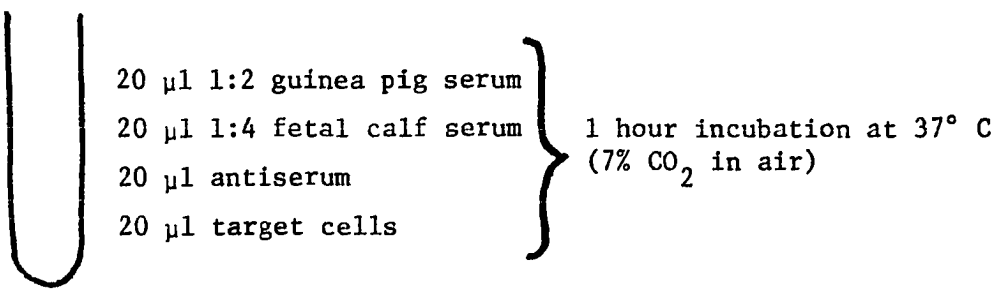
In each assay, 20 µl of the cell suspension (6.0 x 10^6 cells/ml) were mixed with 20 µl of the appropriate serum dilution and 20 µl of a 1:4 dilution of fetal calf serum in 12 x 75 mm glass tubes. Then 20 µl of a 1:2 dilution of guinea pig serum (Miles Laboratory) were added as the source of complement. The guinea pig serum had been absorbed previously with agarose (Cohen and Schlesinger, 1970). The mixture was allowed to incubate at 37° C for one hour in 7% CO_2 in air, after which 20 µl of a 0.4% filtered trypan blue solution were added to each tube. After three to five minutes, a minimum of 200 cells was assessed microscopically for viability. Samples were counted using a hemocytometer (American Optical

Protocol 1



Add 10 µl of 0.4% trypan blue dye solution and score microscopically.

Protocol 2



Add 20 µl of 0.4% trypan blue dye solution and score microscopically.

Figure 2. The trypan blue dye exclusion assay

Corporation, Buffalo, New York) and a binocular microscope (Wild, Heersbrugg, Switzerland or American Optical Corporation) adjusted to low power phase-contrast or bright field. Each assay was performed in duplicate.

3. RNA synthesis assay

Antibody mediated cytotoxicity to spleen cells was evaluated by measuring RNA synthesis in target cells after treatment with antiserum and complement. The protocol is summarized in Figure 3. In each assay, 20 µl of the 6.0 x 10^6 cells/ml suspension were combined with 20 µl of the appropriate serum dilution, 20 µl of a 1:4 dilution of heat-inactivated fetal calf serum, and 20 µl of a 1:2 dilution of absorbed guinea pig serum. The assays were performed in 12 x 75 mm culture tubes fitted with caps (Falcon, Oxnard, California). If the procedure were automated, using an automatic cell harvester, the assays could be performed in 96-well microtiter plates. Tubes were incubated at 37° C in 7% CO_2 in air for one hour. After this one hour preincubation, 25 µl (500 µ Ci/ml) of a [^{3}H]-uridine (26.7 Ci/mmole, New England Nuclear, Boston, Massachusetts) solution were added. The radioactive solution was prepared prior to addition by diluting the [^{3}H]-uridine stock solution (1.0 mCi/ml) with an equal volume of culture medium. Simultaneously, 20 µl of supplemented culture medium were added to each tube. The supplemented culture medium served to maximize viability during labeling, and also to ensure a final volume of 150 µl for spotting on filter papers, as described below.

After addition of the [^{3}H]-uridine, the cells were cultured for an additional five hours, after which 25 µl of a solution containing yeast

RNA (8 mg/ml) and 12% (w/v) deoxycholate in PBS or water (45° C) (See Table 3) were added. The contents of the tubes were vigorously vortexed and spotted in 75 µl aliquots on DEAE-cellulose paper discs (Whatman DE81, 23 mm diameter, Clifton, New Jersey). Each tube was washed twice with 75 µl of a 0.01 M Tris, pH 7.4, 0.5% BSA solution (Table 3) and the washings spotted on two additional filter papers. Duplicate blanks consisted of tubes containing all ingredients, except spleen cells.

The filters were then processed in a batch (the filters were numbered in pencil for identification) by the method of Litman (1968) as modified by Versteegh et al. (1975). The principle of the procedure is that DEAE-filter papers retain RNA by electrostatic attraction and matrix effects, while low molecular weight material (less than 10 nucleotides) is easily removed by extensive washing in phosphate buffer. The advantages of this procedure over conventional trichloroacetic acid precipitation on glass filter papers are that blank values can be reduced to less than 0.1% of the added radioactivity. Also, no unincorporated label is occluded by a large precipitate, and no high molecular weight material is removed by extensive washing. This means that recovery of small samples is quantitative and highly reproducible.

Thus, the filters were washed extensively in 0.5 M sodium phosphate, pH 9.2 (Table 3), to remove unincorporated uridine. Following the final wash, the filters were quickly rinsed twice with distilled water, twice with 95% ethanol, once with ether, and finally air dried. They were counted in a Packard 2405 Liquid Scintillation counter (Downer Grove, Illinois) using

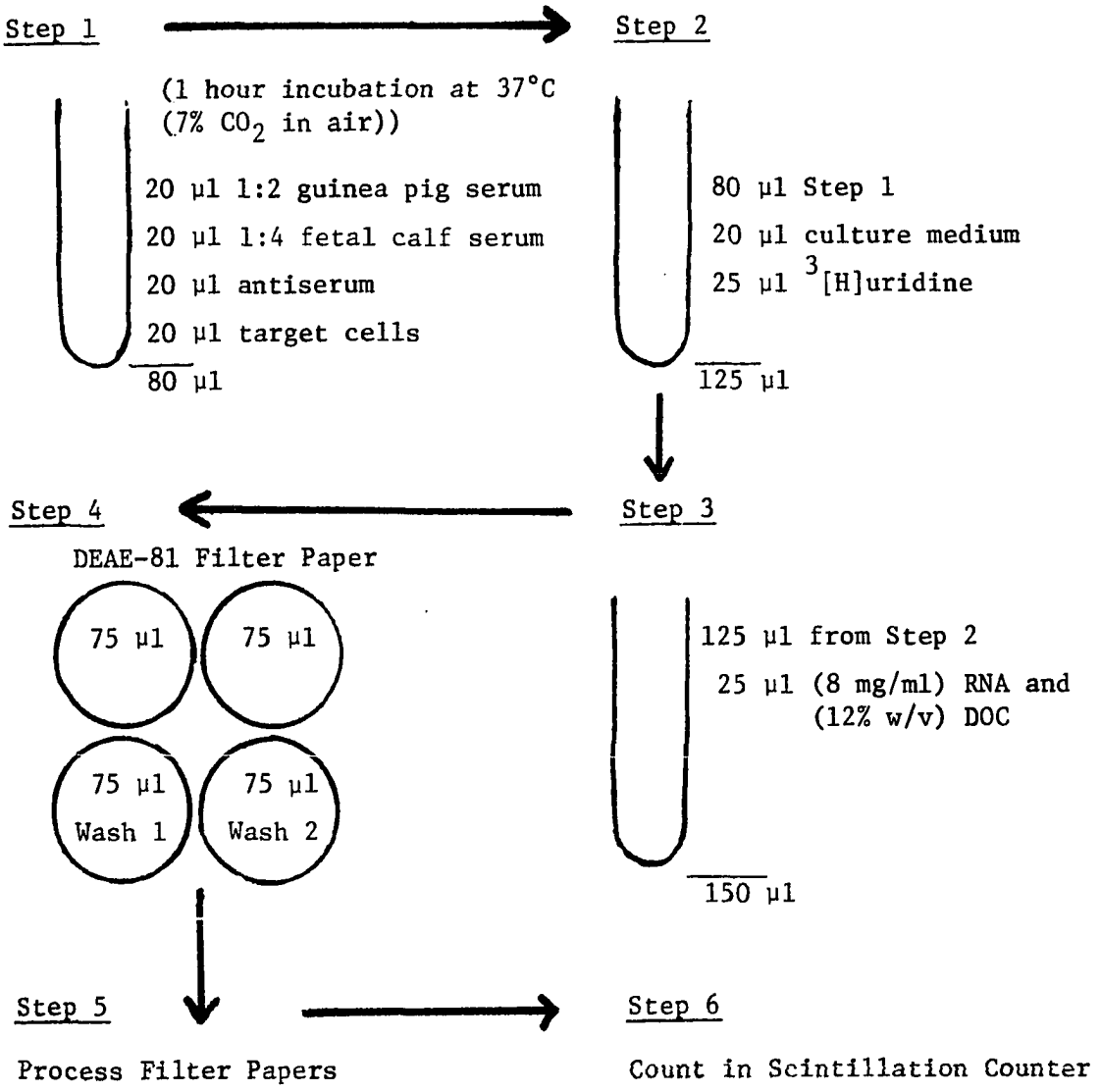


Figure 3. The RNA synthesis assay

10 ml of a toluene counting solution containing 18.6 g 2,5-diphenyloxazole (PPO) and 0.94 g p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) per gallon of toluene. The filter papers may be removed from the vials and the fluor reused several times.

a. Cytotoxic quantitation The percentage of dead cells was calculated using the following formula:

$$P = \frac{\left[1 - \frac{S-B}{T-B}\right]}{\left[1 - \frac{C-B}{T-B}\right]} \times 100$$

where:

P = percentage of dead cells in an assay;

B = average cpm in blank tubes containing no spleen cells;

S = average cpm in tubes containing an unknown sample of spleen cells and antiserum;

C = average cpm observed in tubes with a theoretical 100% killing with antiserum. If the antiserum actually gives 100% killing of cells, then C = B, and the denominator drops out of the equation; and

T = average cpm in control tubes containing spleen cells and normal mouse serum.

b. Experimental protocols To compare the sensitivity and accuracy of the RNA synthesis assay to the trypan blue dye exclusion assay, two types of experiments were designed. In the first type, the five antiserum samples were titered using a constant number of target cells. Supplemented

culture medium was used as diluent for the antiserum samples. Increasing serum dilutions were chosen to reflect changes in cytotoxicity and to permit the contruction of cytotoxic killing curves for each of the antiserum samples. Cytotoxicity was monitored using both the RNA synthesis assay and the trypan blue dye exclusion assay.

In the second type of experiment, the antiserum concentration was kept constant, and the target cell concentration was varied. It should be noted that the RNA synthesis assay measures the number of live cells in a population directly, regardless of the number of dead cells present. Therefore, various dilutions of spleen white blood cells were made with supplemented RPMI 1640. Cells were treated with normal serum and complement for one hour and the RNA synthesis activity measured, as previously described. The observed counts per minute were used as an index of the number of live spleen white blood cells in a culture. In contrast, the trypan blue dye exclusion assay measures the number of dead (or live) spleen white blood cells relative to the total number of cells present. Thus, to get directly comparable results between the two assay procedures, the trypan blue dye exclusion assay was set up as a series of mixing experiments. For example, A spleen white blood cells and C57BL/6 spleen white blood cells were mixed in varying proportions so that the total cell number in each assay would be constant. Thus, a culture tube might contain 9.6×10^4 A cells and 2.4×10^4 C57BL/6 cells, while another might contain 6.0×10^4 A cells and 6.0×10^4 C57BL/6 cells. The total cell number in each assay was 12×10^4. Cells were treated with the appropriate antiserum and complement and the percentage of dead cells determined.

The cells selected for mixing experiments were chosen to minimize nonspecific cytotoxicity of each antiserum sample with the other cell type. The percentage of dead cells killed by a specific antiserum would reflect the number of cells of one type in a mixture at the beginning of the experiment, and the results would be directly comparable to the viable cell number determined by the RNA synthesis assay. Various cell numbers of five different inbred strains of mice were analyzed using both the RNA synthesis assay and the trypan blue dye exclusion assay.

E. Analysis of Hemoglobin

1. Preparation of hemoglobin

Hemoglobins from the inbred strains and allophenic mice (Table 2) were collected using the modified method of Clegg and Schroeder (1959). The procedure is diagrammed in Figure 4. In the experiments, 0.10 ml of whole blood was collected from the orbital venous sinus of each mouse using a 5 3/4-inch pasteur pipet and rapidly mixed with 0.05 ml of 3.2% sodium citrate (pH 5.5) at 4° C. Murine erythrocytes were then washed in 0.85% saline (pH 7.0) and centrifuged (Beckman Microfuge, 0.4 ml tubes) for one minute. After three successive washings and centrifugations, cells were lysed with distilled water and centrifuged for two five-minute intervals, discarding pelleted cell stroma between centrifugations. Hemoglobins were treated with carbon monoxide (5-10 psig) for one and one-half minutes and stored at -70° C (Beckman Microfuge, 0.4 ml tubes) or -196° C (Provial Cooke Laboratory Products, Alexandria, Virginia, 2.0 ml tubes).

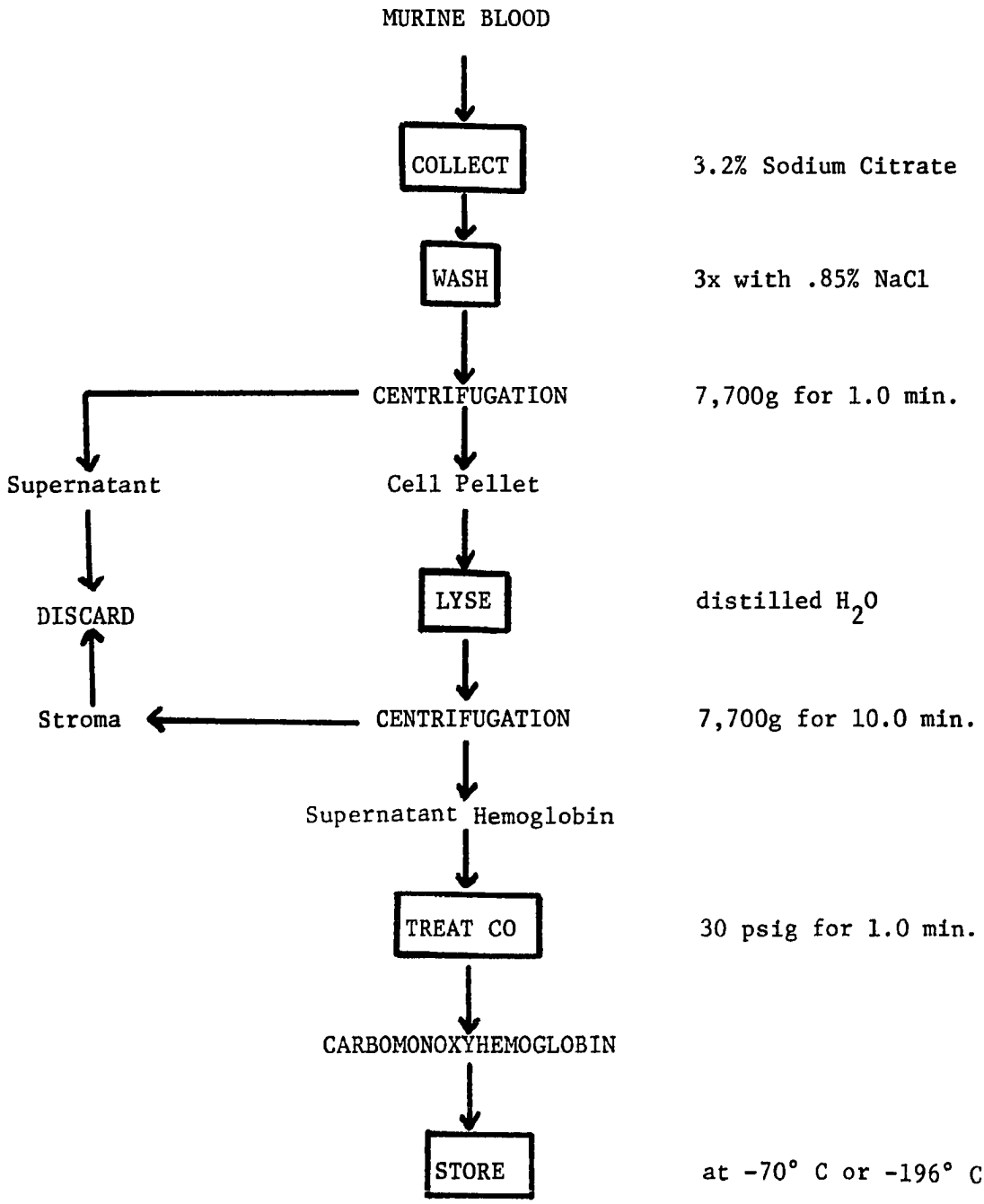


Figure 4. Flow diagram of the modified procedure of Clegg and Schroeder (1959) used for the preparation and storage of hemoglobin

Hemoglobins were prepared for polyacrylamide gel isoelectric focusing (PAGIF) by coupling an S-ethylamine group to the accessible sulfhydryl groups, thus effecting resolution of the structurally different hemoglobins (Smithies, 1965; Wegmann and Gilman, 1970). First, hemoglobin concentrations were determined by diluting 10 µl aliquots of samples with 0.99 ml distilled water in 12 x 75 mm glass test tubes. Samples were vortexed and concentrations determined spectrophotometrically in a Beckman D.U. spectrophotometer by reading absorbance at 576 nm (Figure 5). The samples were diluted with distilled water to a concentration of 20 mg/ml. To this was added an equal volume of reducing buffer (See Table 3). Modification of the hemoglobin was caused by the addition of dry cystamine dihydrochloride (Sigma Chemical Company, St. Louis, Missouri) to a final concentration of 0.25 M. Samples were diluted to a final concentration of 5.0 mg/ml with an equal volume of sucrose-cyanide diluting buffer pH 8.7 (see Table 3). The exact concentration of the hemoglobins was verified by the absorption at 576 nm ($\varepsilon = 3.77 \times 10^4\ M^{-1}\ cm^{-1}$) previous to sample application to gels. When artificial mixtures of the structurally different hemoglobins were used, the mixtures were prepared before application to the gels.

2. Polyacrylamide gel isoelectric focusing (PAGIF)

The technique of polyacrylamide gel isoelectric focusing was a modification of a previously described method (Drysdale et al., 1971; Righetti and Drysdale, 1972) using a Buchler Polyanalyst equipped

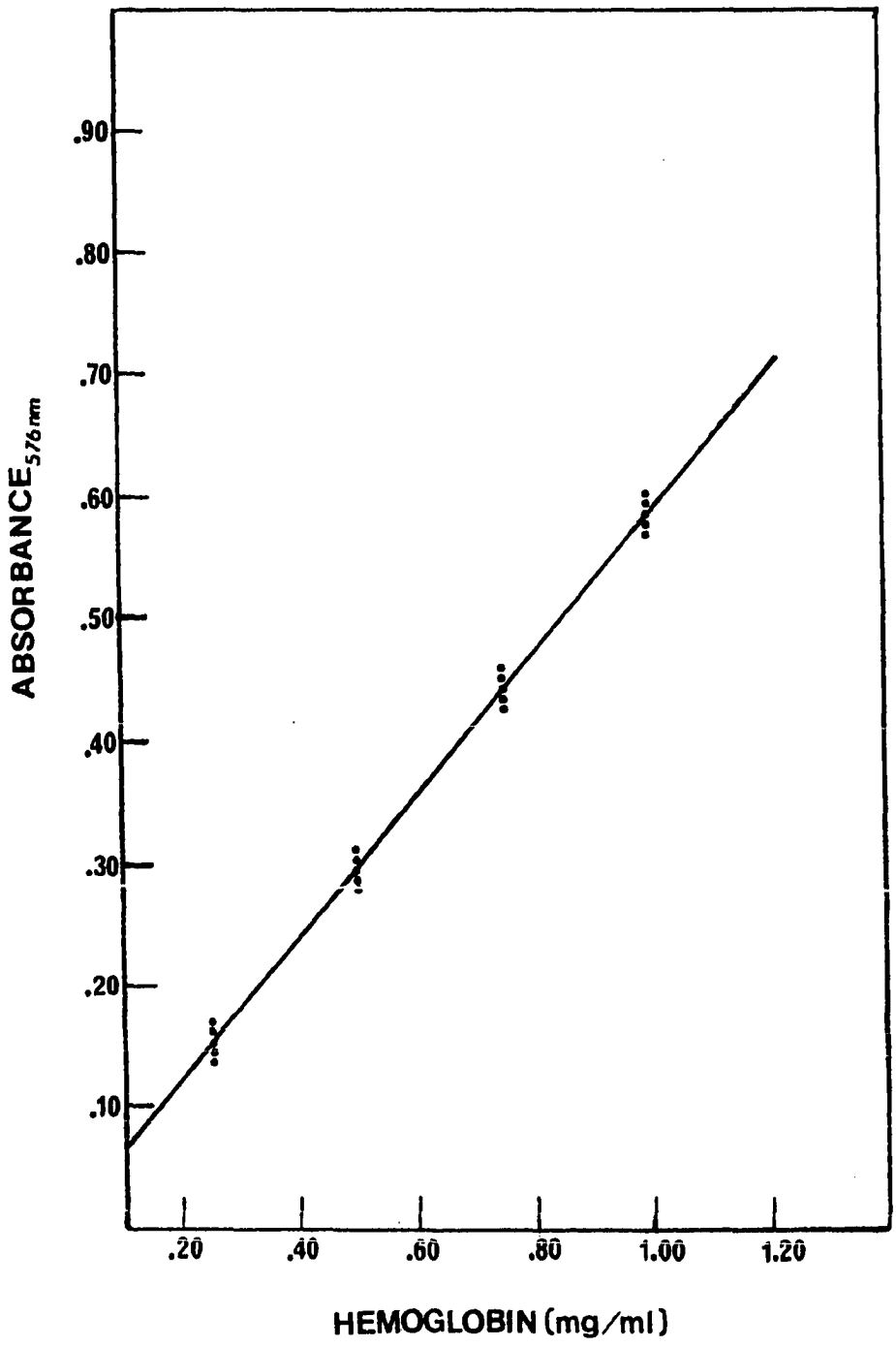


Figure 5. Standard curve used to determine protein concentration of hemoglobin samples from inbred, F_1 hybrids, and allophenic mice. [The accuracy of the curve was verified by Lowry et al. procedure of protein determination (1951).]

with a Lauda circulator pump (West Germany) preset at 4° C and a Buchler regulated power supply.

The gels were prepared as follows. In a 50 ml beaker, 8.17 ml of an acrylamide and methylene-bis-acrylamide (Bio-Rad, Richmond, California) stock solution (292.5 g/l acrylamide and 14.6 g/l methylene-bis-acrylamide) were mixed with 1.40 ml of glycerol, 22.2 ml of distilled water, and 0.53 ml of ampholytes (3-10) (Bio-Rad), 0.53 ml of either ampholytes (6-8) (Bio-Rad) or ampholytes (5-8) (LKB), and 2.24 ml of a solution containing 1% (v/v) TEMED (Bio-Rad) and 0.01% (w/v) riboflavin 5'phosphate (Bio-Rad). After mixing, gel tubes (12 x 0.5 cm) were filled within 2 cm of the top, after which water was carefully layered on top of the gel solution. Any occasional inconsistencies in gel polymerization, when using different preparations of ampholytes, were corrected by increasing the volume of the (3-10), (6-8) ampholytes to 1.0 ml, respectively, while decreasing the volume of distilled water to 21.2 ml. The volume of other gel components remained unchanged. Gels were photopolymerized for 10 hours and excess water removed before sample application. Three hundred fifty milligrams of artificial mixtures of mouse hemoglobins or allophenic mouse hemoglobins were applied to the surface of the gels. To safeguard against possible denaturation of proteins at the interface by the catholyte, 100 µl of 20% and 100 µl of 10% sucrose were overlayed on the sample. Solutions (300 ml) of 0.02M phosphoric acid (pH 2.2) and 0.01M sodium hydroxide (pH 12.0) were used as anolyte and catholyte, respectively, with the catholyte uppermost (see Table 3). A current of 1 mA/tube was established until the voltage

rose to 500 V. Thereafter, the voltage was maintained at this level for the duration of the experiment. Isoelectric focusing was complete within four hours (Dale and Latner, 1968; Righetti and Drysdale, 1972). Gels were then removed from their tubes and washed in 10% TCA (See Table 3) for one and one-half hours, to fix the protein and remove the ampholytes (Dale and Latner, 1968). The procedures of hemoglobin modification and polyacrylamide gel isoelectric focusing are diagrammed in Figure 6.

3. Hemoglobin quantitation

Gels were scanned at a wavelength of 576 nm using either a Zeiss Spectrophometer and Strip Chart Recorder (Oberkochen, Germany) or a Beckman ACTA Spectrophotometer (Fullerton, California) and a Heath Strip Chart Recorder (Model EU-200-01) (Benton Harbor, Michigan) preset at a 10 mV with a chart speed of 15 sec/cm. Total area under the curves was established by tracing with a polar planimeter (K and E, New York) or by cutting out and weighing recorded gel scans. Areas under individual peaks were resolved using the following formula (Disc Instruments, 1973).

$$P_1 = \frac{h_1}{h_1 + h_2 + h_3} \times (\text{Total Area})$$

$$P_2 + P_3 = \text{Total Area} - P_1$$

where:

P_1 = area under largest peak;

P_2 = area under intermediate peak;

P_3 = area under smallest peak;

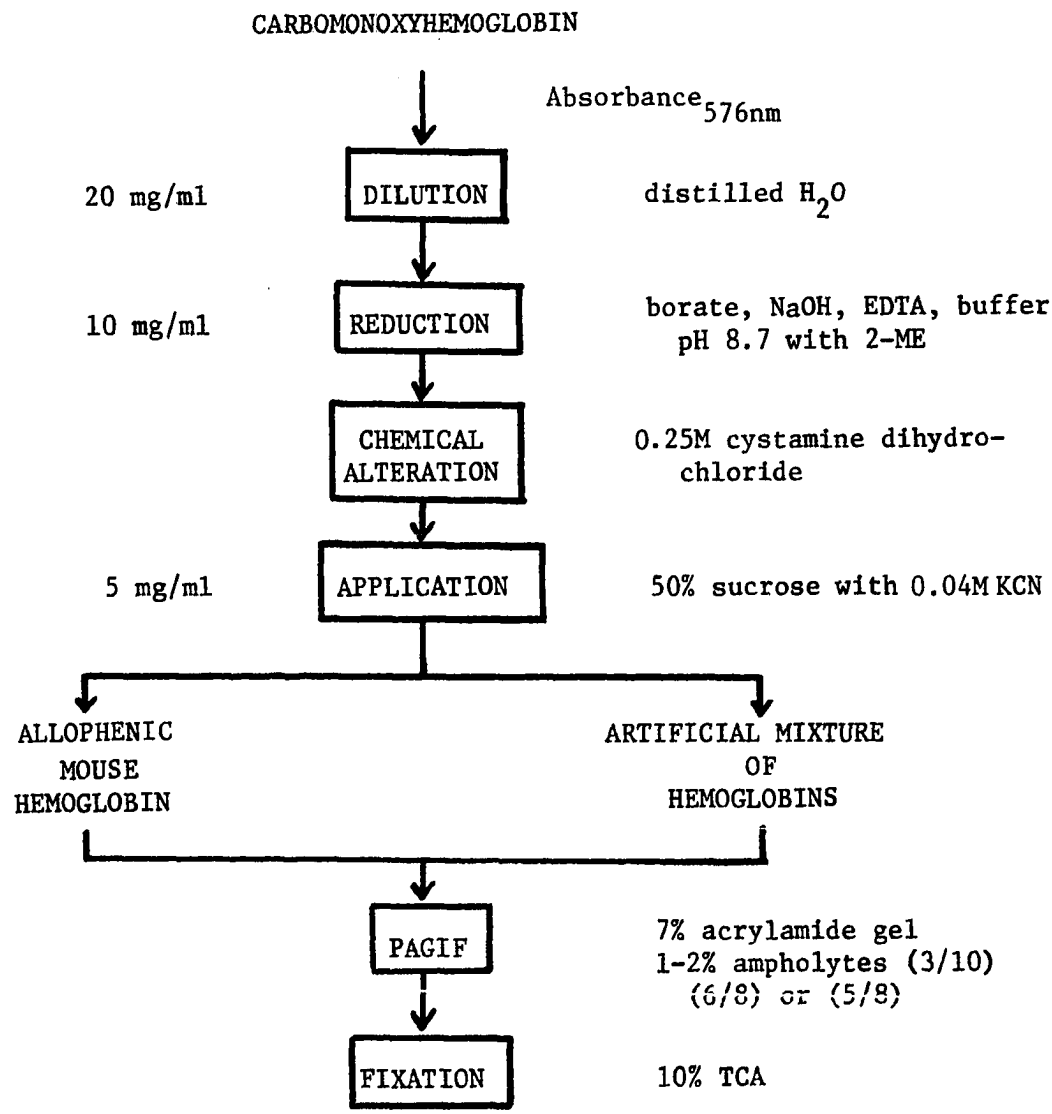


Figure 6. Flow diagram of hemoglobin modification technique (Smithies, 1965; Wegmann and Gilman, 1970) and PAGIF procedures (Drysdale et al., 1971; Righetti and Drysdale, 1972) used to resolve artificial mixtures of allophenic mouse hemoglobins

h_1 = height of largest peak;

h_2 = height of intermediate peak; and

h_3 = height of smallest peak.

Composition of unknown mouse hemoglobin was estimated relative to artificial parental strain hemoglobin mixtures. To overcome differences among strains of the proportionality of the area of each peak to its height, a set of at least four artificial mixtures was included with each set of allophenic mouse hemoglobins for the C57BL/6 ⟷ (AXSJL)F_1, (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 and C57BL/6 ⟷ DBA/1 mice. The composition of the hemoglobin of the C57BL/6 ⟷ A and C57BL/10Sn ⟷ A mice was determined by comparison to separate standard curves established during their respective experiments. All hemoglobin analyses for a particular allophenic mouse were run simultaneously. A duplicate run was made at a later time. The standard curves were established by drawing the least-squares lines through the areas of the curves in the artificial mixtures versus the known amount of hemoglobin applied to the gels. For the (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, C57BL/6 ⟷ DBA/1, C57BL/6 ⟷ A, and C57BL/10Sn ⟷ A, this plot was always in terms of the percentage of C57BL/6 or C57BL/10Sn hemoglobin in the mixtures. For the C57BL/6 ⟷ (A x SJL)F_1 mice, this plot was always in terms of the percent A in the mixtures since C57BL/6 hemoglobin and SJL hemoglobin are indistinguishable on isoelectric focusing (see Results).

4. Determination of isoelectric points

The isoelectric point (pI) of hemoglobin samples, from six inbred strains of mice and (A x SJL)F_1 hybrids, was determined before and after modification with cystamine dihydrochloride using an adaptation of the procedures of Righetti (1974). Untreated hemoglobins were prepared by diluting samples with distilled water to a concentration of 20 mg/ml to which an equal volume of buffer (0.2 M boric acid, 0.08 M NaOH and 0.01 M EDTA) was added. These samples were not treated with 2-ME or dry cystamine dihydrochloride. Chemically modified hemoglobins were prepared using techniques previously described (Stephens et al., 1977; Warner, et al., 1977d). Both unmodified and modified hemoglobins were diluted to a final concentration of 5.0 mg/ml with an equal volume of sucrose-cyanide diluting buffer, pH 8.7 (see Table 3). To minimize differences between runs, the same preparations of ampholytes were used and samples were electrophoresized under identical conditions. After isoelectric focusing was complete, gels were removed from their tubes and their length and hemoglobin banding pattern were measured and recorded. Any slight variations between duplicates were corrected by correlating banding patterns with the actual pH gradients developed in experiments (Righetti, 1974).

Gels were then quick frozen in liquid nitrogen and sliced into 5.0 mm sections with an apparatus similar to the Biorad Model 190 Gel Slicer. Slices from duplicate gels were placed in 12 x 75 mm glass test tubes and ampholytes were eluted with 1.0 ml distilled water containing 1 mM sodium chloride. The water was boiled prior to its addition to tubes to

minimize absorption of atmospheric carbon dioxide (Righetti, 1974). After cooling to room temperature, the pH of the gel eluates was measured with a pH meter (The London Company, Cleveland, Ohio) equipped with a combination electrode.

Measured pH values from identical gel slices were averaged and a standard curve was established by drawing a least-squares line through the pH values which varied as a function of gel length. The constructed pH gradient then served as a standard curve from which isoelectric points of hemoglobin samples could be estimated.

F. Preparation of Frozen Lymphocytes

1. Cell preparation

Spleen cells and thymocytes from allophenic and inbred mice were prepared for long-term storage using an adaptation of the freezing procedures of Fotino et al. (1967) and Bates and Sell (1970). Briefly, spleens and thymuses were prepared into single cell suspensions (Section D. Antibody Mediated Cytotoxicity Testing, Part 1. Cell preparation) and purified by Ficoll-Hypaque density gradient centrifugation (Böyum, 1968). The white blood cell concentration was adjusted to 2.0×10^6 cells/ml using L-15 medium with 10% heat-inactivated fetal calf serum and cells were maintained at 4° C in an ice bath.

Cells were prepared for freezing as follows. To a 50 ml flask containing 2-15 ml of 2.0×10^6 cells/ml, an equal volume of 14% (w/v) dimethylsulfoxide solution (DMSO) (4° C) was added slowly to cells with

constant swirling of the flask. The DMSO was diluted with phosphate buffered saline, pH 7.0 prior to usage (see Table 3). All reagents were maintained at 4° C throughout the experiment to prevent temperature dependent cytotoxicity (Malinin and Perry, 1967). Ten minutes after the addition of the DMSO, the lymphocyte suspension was pipetted (1.0 aliquots) into either 2 ml-size glass ampoules or plastic 2 ml Provials (Cooke Laboratory Products) prechilled at 4° C. Ampoules and vials were color coded and labeled with a designated mouse number and cell type.

The final cell concentration in a container was 1.0×10^6 cells/ml in 7% DMSO (w/v).

2. Cell freezing procedure

The freezing of biological specimens was conducted with a Linde 4-2 Biological Freezing System (Union Carbide, New York) composed of a BF-6 controller, BF4-2 freezing chamber, liquid nitrogen tank (22 psig) and a Bristol Dynamaster Recorder, model IPH 560-51 (Waterbury, Connecticut) with an adjusted chart speed of one inch per minute.

After a 15-30 minute warm-up period, the recorder was calibrated by placing the differential thermocouple probe into beakers containing 25 ml of distilled water at 25° C, 15° C, 10° C, and 5° C, respectively. During the experiments, actual cooling rates (degrees per minute) were calculated by dividing ΔT, the differential temperature change, by ΔC, the chart speed. A typical change was one degree Celsius per one inch of paper.

A control vial containing 1.0 ml of 1.0×10^6 cells/ml in 7% DMSO was connected to the differential thermocouple and inserted into the

freezing chamber. The controller was adjusted to a setting of 8 which roughly corresponded to a freezing rate of 1° C per minute. The chamber was cooled to 5° C, the samples were distributed evenly into the freezing chamber, and frozen at a controlled rate of 1° C per minute. When the specimens were 1° to 4° C above their freezing point (-12° C), liquid nitrogen was allowed to flow uninterrupted into the chamber for 30-40 seconds, thereby minimizing supercooling and the random freezing of samples (Bates and Sell, 1970).

The freezing of biological samples was continued at a controlled rate of 1° C per minute to -30° C and then 10° C per minute to -70° C. Vials were then rapidly transferred to and stored in a cryogenic refrigerator, model LR-21 (Union Carbide) filled with liquid nitrogen. Samples were maintained in the refrigerator for a one to two year period.

3. Thawing of lymphocytes

Ampoules and vials containing lymphocytes were removed from the cryogenic refrigerator and rapidly thawed by agitation in a water bath at 40° C. The contents of the containers were gently transferred to a 17 x 100 mm plastic test tube (Falcon) to which washing solution was added dropwise from a 25 ml buret, while constantly agitating. Washing solution was added in ratio of four parts to one part cell volume (see Table 3). After washing, cells were centrifuged at 1,100 rpm (180 g) for 10 minutes in a refrigerated International PR-2 centrifuge and resuspended to a concentration of 1-6.0 x 10^6 cells/ml in supplemented RPMI 1640 (see Table 3).

Before using frozen-thawed cells in experiments, viability was evaluated by diluting a 10 µl aliquot of cells 1:3 with supplemented culture medium. To this was added 10 ul of a 0.4% filtered trypan blue solution. After five minutes a minimum of 200 cells were assessed microscopically for viability.

G. Testing for Chimeric Serum Blocking Factors

1. Allophenic mouse serum and allophenic spleen white blood cells

The effectiveness of allophenic mouse sera in blocking antibody mediated complement dependent cytotoxicity was tested on frozen-thawed spleen white blood cells (Section F. Preparation of Frozen Lymphocytes). Both the cells and the allophenic sera had been frozen one to two years at the time of these experiments. Only target cells showing 70% or greater viability two hours following thawing were used for these experiments. Dead cells were not removed from the population. (Ficoll-Hypaque isolation of dead cells used on human lymphocytes did not work on the mouse lymphocytes) (Davidson and Parish, 1975).

In a preassay step, 25 µl of allophenic target cells (1-6.0 x 10^6 cells/ml) were mixed with 25 µl of a dilution of heat-inactivated allophenic mouse serum in a 12 x 75 mm glass test tube (Tube 1, Figure 7a). Simultaneously, 25 µl of a dilution of anti-spleen or anti-H-2 serum was mixed with 25 µl of a dilution of heat-inactivated allophenic mouse serum (Tube 2, Figure 7a). Both sets of tubes were incubated at 37° C in 7%

CO_2 in air for one hour. As a control (see Figure 7b), 25 µl of an equal mixture of the appropriate parental type normal sera were added to a 25 µl aliquot of target cells (Tube 4, Figure 7b) and a 25 µl aliquot of antiserum (Tube 5, Figure 7b) and incubated as above. Both the allophenic mouse serum and mixed parental serum were filtered with a .45 µm disposable millipore filter unit (Millipore Co., Bedford, Massachusetts) and diluted with supplemented RPMI 1640 prior to experiments.

Following the one hour preincubation, blocking activity was evaluated using the trypan blue dye exclusion assay (Section D. Antibody Mediated Cytotoxicity Testing, Part 2. Trypan blue dye exclusion assay).

Briefly, as shown in Figures 7a,b in each assay 20 µl of the serum treated target cell suspension (Tube 1 or Tube 4, Figures 7a,b) were mixed with 20 µl of serum treated antiserum (Tube 2 or Tube 5, Figures 7a,b) and 20 µl of a dilution of allophenic mouse serum or control mixed parental serum were added. Then 20 µl of a 1:2 dilution of agarose absorbed guinea pig serum were added as a source of complement. The mixture (Tube 3 or Tube 6, Figures 7a,b) was allowed to incubate at 37° C for one hour in 7% CO_2 in air, after which 20 µl of a 0.4% filtered trypan blue solution was added to each tube. After five minutes a minimum of 200 cells were assessed microscopically for viability. Each assay was performed in duplicate and the percentage of dead cells calculated. Duplicate tubes containing only allophenic spleen cells and supplemented RPMI 1640 served as controls to monitor cell death resulting from thawing.

For each allophenic mouse, samples of cells from the two parental strains comprising the particular mouse were included as controls. This

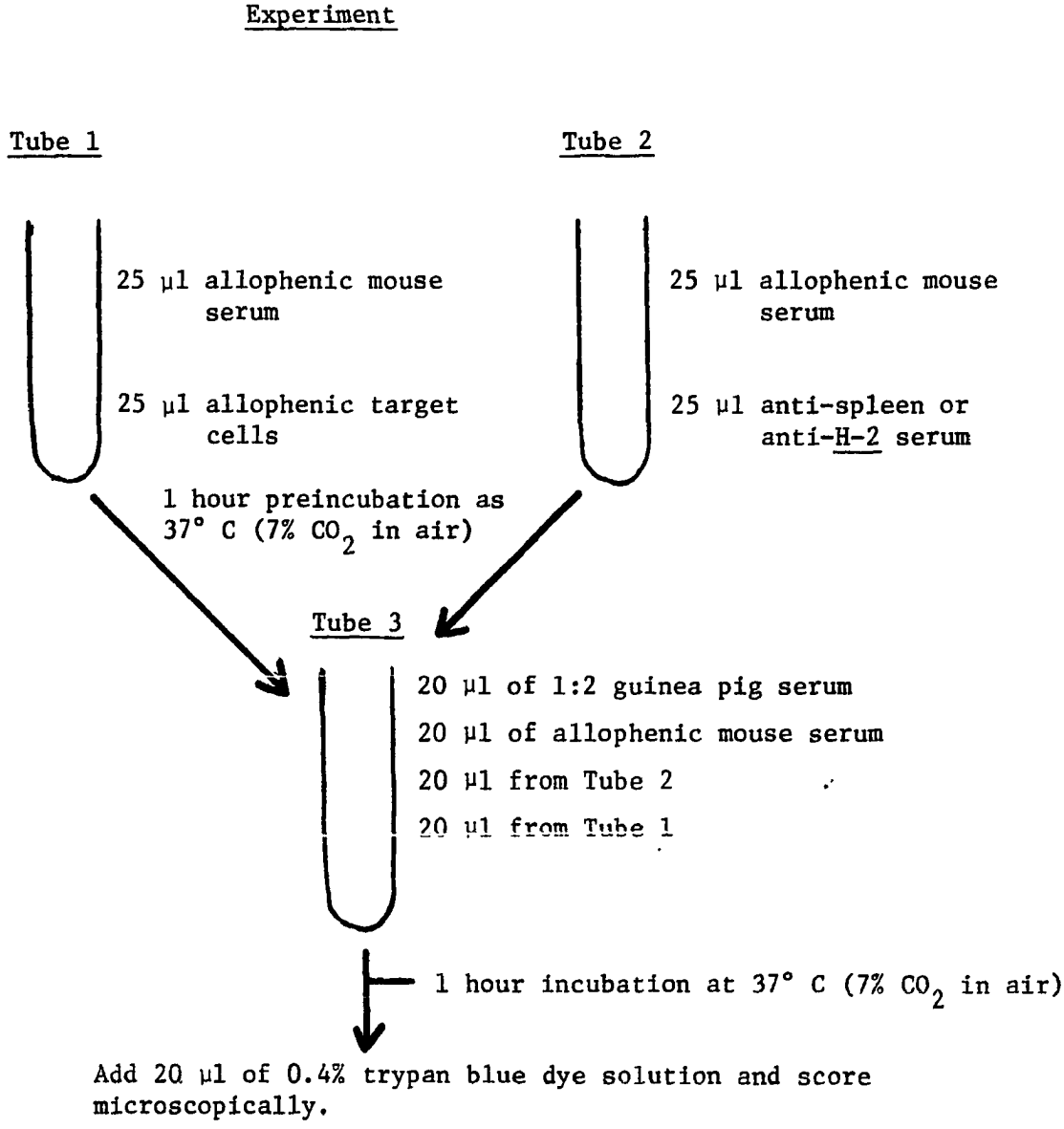


Figure 7a. The effect of allophenic mouse serum on allophenic target cells

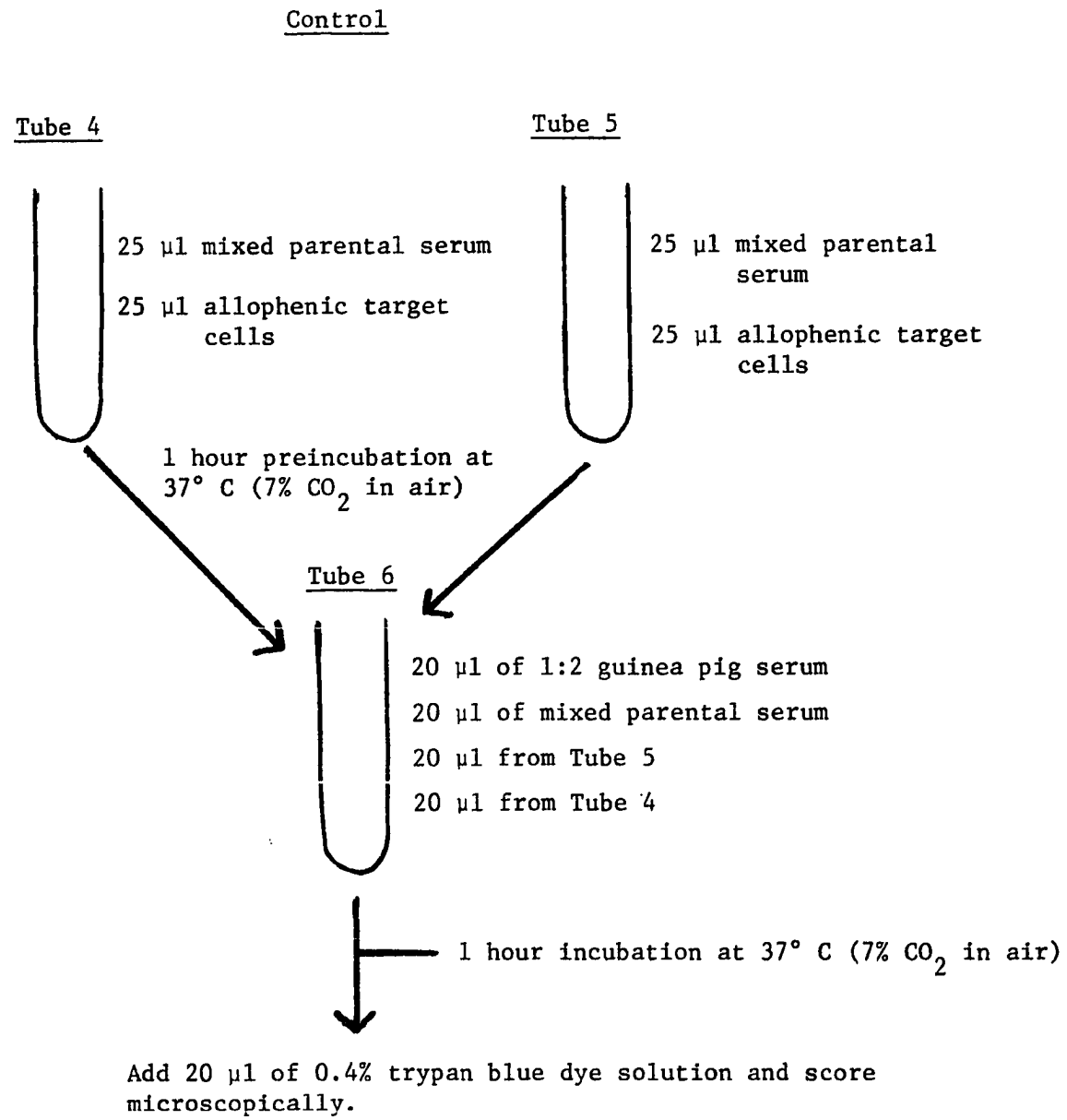


Figure 7b. The effect of mixed parental serum on allophenic target cells

occasionally resulted in values greater than 100%. For instance, if a sample of anti-C57BL/6 serum showed 95% killing of the C57BL/6 cells and 99% killing of the allophenic mouse cells of unknown composition, this value would be corrected to give 104% C57BL/6 as the composition of the allophenic mouse.

2. Allophenic mouse serum and allophenic thymocytes

The blocking activity of allophenic mouse sera was evaluated against frozen-thawed allophenic thymocytes using the protocol described above (Figures 7a,b). Anti-thymocyte sera, prepared by the reciprocal immunization protocol were used to kill thymocytes. As before, data for each mouse were corrected to the killing of the fresh thymocyte control, which occasionally resulted in values exceeding 100%.

3. Allophenic mouse serum and parental spleen white blood cells

The possible blocking activity of allophenic mouse sera was tested with fresh parental spleen white blood cells in the RNA synthesis assay (Section D. Antibody Mediated Cytotoxicity Testing, Part 3. RNA synthesis assay) using a 50% cytotoxic dose of antisera. The protocol is described in Figures 8a,b.

In the preincubation step, 25 µl of a spleen cell population (6.0 x 10^6 cells/ml) were mixed with 25 µl of a 1:5 dilution of heat-inactivated allophenic mouse serum in a 12 x 75 plastic culture tube (Falcon) (Tube 1, Figure 8a). Simultaneously, 25 µl of a dilution of anti-spleen serum

was selected to give approximately 50% cytotoxic endpoint upon mixing with the allophenic mouse serum. The 50% endpoint was determined previously using the trypan blue dye exclusion assay. Both sets of tubes were incubated for one hour at 37° C in 7% CO_2 in air. In the control experiments (Figure 8b), either 1:5 normal parental or mixed parental serum (mixed in an equal ratio) was mixed with target cells or anti-spleen serum and incubated under identical conditions (Tubes 4 and 5, Figure 8b). Blocking activity was evaluated using RNA synthesis as a probe of antibody mediated cytotoxicity. In each tube (Tubes 3 and 6, Figures 8a,b) 20 µl of a 1:5 dilution of allophenic mouse serum or normal parental mouse serum replaced the 1:4 dilution of fetal calf serum described in the RNA synthesis assay protocol. The percentage of dead cells was calculated using the mathematical formula previously described (Section D. Antibody Mediated Cytotoxicity Testing, Part 3. RNA synthesis assay, Paragraph a. Cytotoxic quantitation).

4. Statistical analysis of data

To determine whether the various serum treatments, applied to target cells and antisera, were significantly effective, least significant differences ("t" test) were performed on the appropriate paired samples at each serum dilution. Samples were evaluated at 0.05 and 0.01 levels of significance. To increase the degrees of freedom all paired samples of serum dilutions tested for a single mouse were pooled. Statistical analysis of some of the data was performed by Drs. David Cox and Mike Hand, Department of Statistics, Iowa State University.

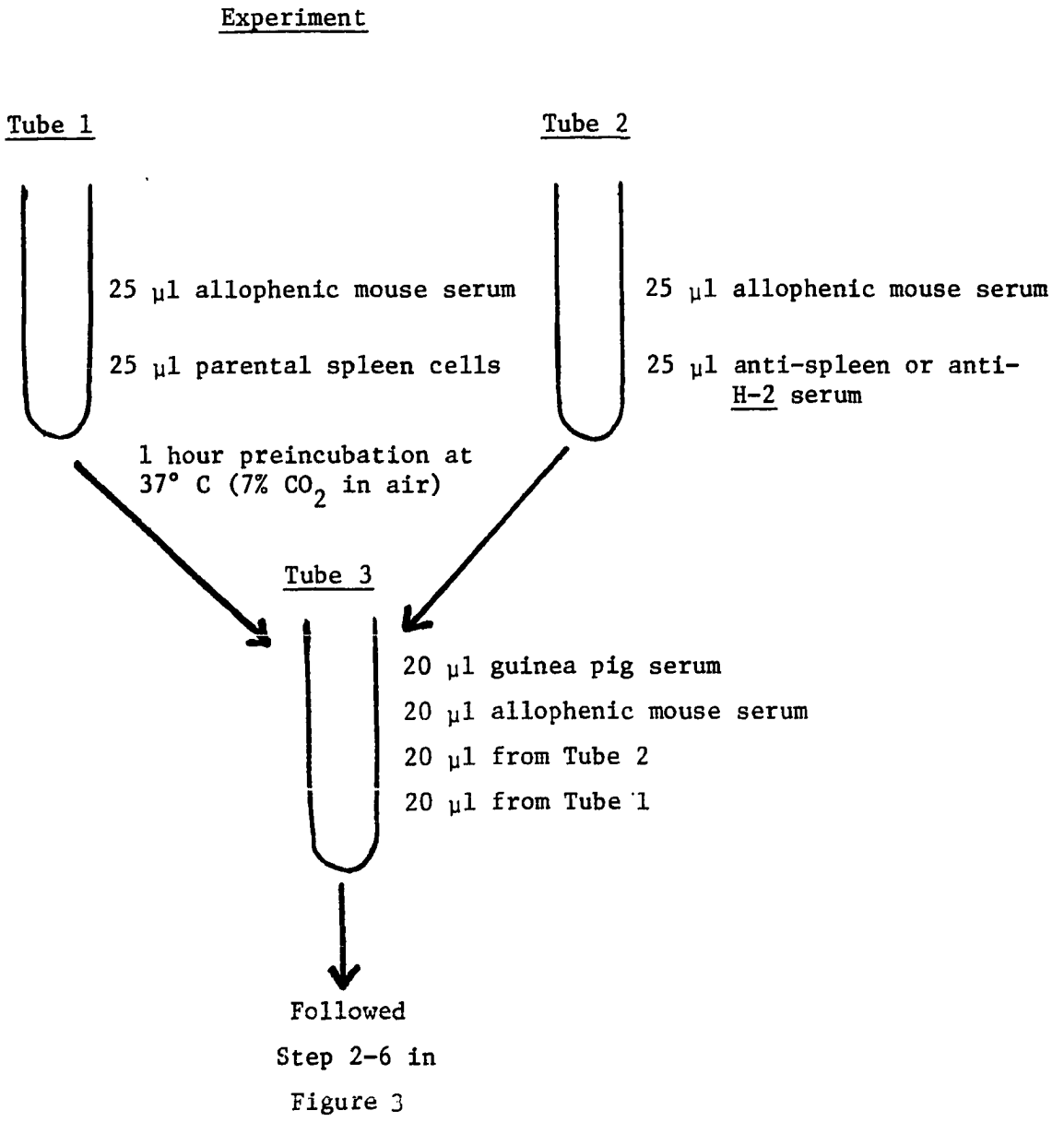


Figure 8a. The effect of allophenic mouse serum on parental target cells

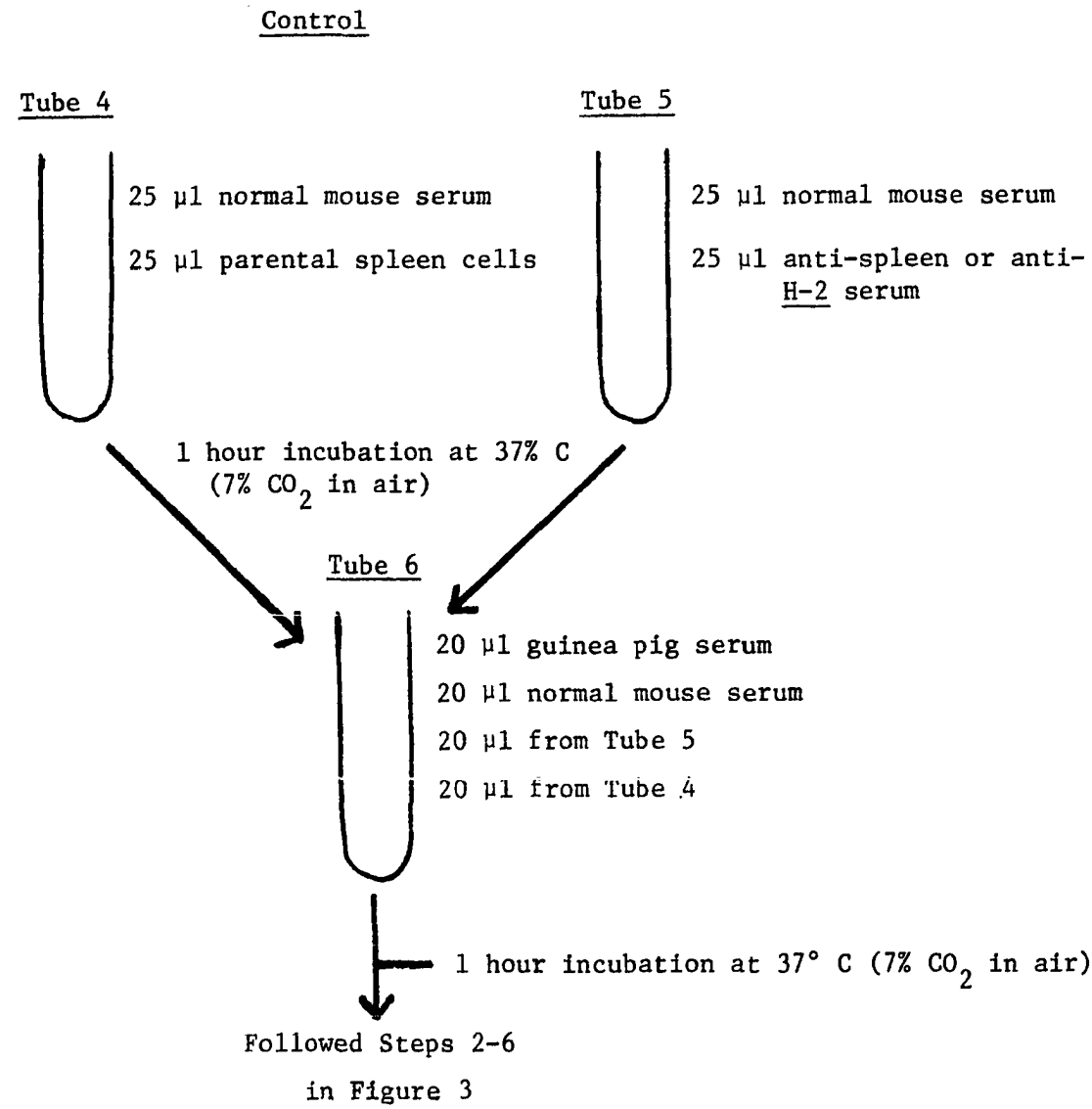


Figure 8b. The effect of normal parental mouse serum on parental target cells

III. RESULTS

A. Allophenic Mouse Production and Usage

A total of 105 allophenic mice were produced in our laboratory for these studies. The mice included 9 different parental combinations as shown in Table 5. Of the 105 mice, 66 were phenotypic males and 39 were phenotypic females. In addition, 48 mice showed both parental coat colors, while 57 mice showed one or the other parental coat color. Data are shown in Table 6 for allophenic mouse descriptions and experimental usages. The experiments in which allophenic mice were examined include cytotoxicity testing for spleen white blood cell, peripheral white blood cell and thymus cell compositions, hemoglobin analysis, chimeric drift, spleen-thymus discordance, the effectiveness of long-term cryogenic storage of spleen white blood cells and thymocytes, and the evaluation of serum blocking factors as a possible mechanism of tolerance in these mice. Tables 5 and 6 utilize the current nomenclature used in our laboratory for describing particular combinations of allophenic mice. Reference to strain 1 is used to indicate the first embryo of the aggregate, while strain 2 refers to the second embryo to give a 1 $\longleftrightarrow$ 2 allophenic mouse.

Table 5. Summary of allophenic mouse production (1974-1977)

Mouse Type[a]	Total Number	Mouse Numbers
C57BL/6 ↔ (A x SJL)F_1	34	83, 84, 85, 86, 87, 98, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 132, 133, 134, 135, 137, 138, 139, 140, 141, 142, 143, 148, 149, 150, 151, 152, 153
DBA/1 ↔ (A x SJL)F_1	3	94, 99, 100
DBA/1 ↔ A	3	182, 183, 186
(CBA x CBA/H-T6)F_1 ↔ DBA/1	17	97, 103, 104, 105, 106, 107, 144, 145, 146, 154, 163, 164, 165, 166, 174, 175, 184
(CBA x CBA/H-T6)F_1 ↔ C57BL/6	15	74, 76, 80, 81, 82, 119, 120, 121, 125, 126, 127, 128, 129, 130, 131
(CBA x CBA/H-T6)F_1 ↔ A	5	177, 178, 179, 180, 181
C57BL/6 ↔ DBA/1	4	101, 122, 123, 124
C57BL/6 ↔ A	15	157, 158, 159, 160, 162, 167, 168, 169, 170, 171, 172, 173, 187, 188, 191
C57BL/10Sn ↔ A	9	205, 206, 207, 210, 211, 212, 213, 214, 215

[a]Mouse type refers to allophenic mice formed by the aggregation of a strain 1 embryo with a strain 2 embryo to give a 1 ↔ 2 allophenic mouse.

Table 6. Allophenic mouse descriptions and experimental usages (1974-1977)

Mouse Number	Parental Combination	Sex	Birth Date	Coat[a] Color	Cytotoxicity Testing[b] SWBC	PWBC	TWBC
74	CBA x CBA/H-T6 ⟷ C57BL/6	♀	7/15/74	S	+	+(2)	-
76	CBA x CBA/H-T6 ⟷ C57BL/6	♀	7/ 9/74	M	+	+(2)	-
80	CBA x CBA/H-T6 ⟷ C57BL/6	♂	7/ 7/74	S	+	+(2)	-
81	CBA x CBA/H-T6 ⟷ C57BL/6	♂	7/ 7/74	S	+	+(2)	-
82	CBA x CBA/H-T6 ⟷ C57BL/6	♂	7/ 7/74	M	+	+(2)	-
83	C57BL/6 ⟷ A x SJL	♂	9/ 1/74	M	+	+(2)	-
84	C57BL/6 ⟷ A x SJL	♂	9/ 1/74	M	+	+(2)	-
85	C57BL/6 ⟷ A x SJL	♀	9/ 1/74	M	+	+(2)	-
86	C57BL/6 ⟷ A x SJL	♂	10/ 7/74	M	+	+(2)	-
87	C57BL/6 ⟷ A x SJL	♀	10/ 7/74	M	+	+(2)	-

[a]Coat color was estimated at the time of weaning. (S) and (M) refer to single and multicolored mice, respectively.

[b]Cytotoxicity testing was performed in duplicate at various times during the life of the mouse and at the time of sacrifice using the trypan blue dye exclusion assay. The abbreviations SWBC and PWBC refer to spleen white blood cells and peripheral white blood cells, respectively. TWBC refers to thymus white blood cells. (+) or (-) indicate whether the composition of the mouse was determined. The number following (+) refers to the frequency of sample collection during the lifetime of the mouse.

[c](+) or (-) indicate whether the hemoglobin composition was determined. The number following (+) refers to the frequency of collection during the lifetime of the mouse.

[d]Changes in hemoglobin (Hb) or peripheral white blood cells (PWBC) composition with time have been called chimeric drift. (+) or (-) indicate whether the mouse was tested.

[e]Difference between the relative proportions of the parental white blood cells in the spleen and thymus is called spleen-thymus discordance. (+) or (-) indicate whether the mouse was tested.

[f](+) or (-) indicate whether spleen white blood cells (SWBC) or thymocytes were stored in liquid nitrogen for a one-two year period.

[g](+) or (-) indicate whether allophenic mouse serum, spleen white blood cells (SWBC) and(or) thymocytes were employed in the examination of serum blocking factors.

Hemoglobin[c] Analysis	Chimeric Drift[d] PWBC	Hb	Spleen Thymus[e] Discordance	Cryogenic Storage[f] SWBC	Thymocytes	Chimeric Serum Blocking[g] Experiments
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-

Table 6. (continued)

Mouse Number	Parental Combination	Sex	Birth Date	Coat Color	Cytotoxicity Testing[b] SWBC	PWBC	TWBC
94	DBA/1 ⟷ A x SJL	♂	10/27/74	M	+	-	-
97	CBA x CBA/H-T6 ⟷ DBA/1	♀	10/27/74	M	+	-	-
98	C57BL/6 ⟷ A x SJL	♂	11/12/74	S	+	+(2)	-
99	DBA/1 ⟷ A x SJL	♂	11/ 9/74	M	+	-	-
100	DBA/1 ⟷ A x SJL	♂	11/ 9/74	M	+	-	-
101	C57BL/6 ⟷ DBA/1	♂	10/27/74	S	+	+(2)	-
103	CBA x CBA/H-T6 ⟷ DBA/1	♂	10/27/74	S	+	-	-
104	CBA x CBA/H-T6 ⟷ DBA/1	♂	10/27/74	M	+	-	-
105	CBA x CBA/H-T6 ⟷ DBA/1	♂	10/27/74	M	+	-	-
106	CBA x CBA/H-T6 ⟷ DBA/1	♀	10/27/74	S	+	-	-
107	CBA x CBA/H-T6 ⟷ DBA/1	♂	10/27/74	S	+	-	-
108	C57BL/6 ⟷ A x SJL	♂	10/27/74	M	+	+(2)	-
109	C57BL/6 ⟷ A x SJL	♂	11/16/74	S	+	+(2)	-
110	C57BL/6 ⟷ A x SJL	♀	11/16/74	S	+	+(2)	-
111	C57BL/6 ⟷ A x SJL	♀	11/16/74	S	+	+(2)	-
112	C57BL/6 ⟷ A x SJL	♂	11/25/74	S	+	+(2)	-
113	C57BL/6 ⟷ A x SJL	♀	11/25/74	M	+	+(2)	-
114	C57BL/6 ⟷ A x SJL	♀	11/25/74	S	+	+(2)	-
115	C57BL/6 ⟷ A x SJL	♀	11/25/74	S	+	+(2)	-
116	C57BL/6 ⟷ A x SJL	♂	12/ 1/74	M	+	+(2)	-
117	C57BL/6 ⟷ A x SJL	♂	12/ 1/74	S	+	+(2)	-
118	C57BL/6 ⟷ A x SJL	♀	12/ 8/74	S	+	+(2)	-
119	CBA x CBA/H-T6 ⟷ C57BL/6	♀	12/ 8/74	M	+	+(2)	-
120	CBA x CBA/H-T6 ⟷ C57BL/6	♀	12/ 8/74	S	+	+(2)	-
121	CBA x CBA/H-T6 ⟷ C57BL/6	♂	12/ 8/74	M	+	+(2)	-
122	C57BL/6 ⟷ DBA/1	♂	10/12/75	S	-	+	-
123	C57BL/6 ⟷ DBA/1	♂	10/12/75	S	+	+(3)	+
124	C57BL/6 ⟷ DBA/1	♂	10/12/75	M	+	+(3)	+
125	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	M	+	+(3)	+
126	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	M	+	+(3)	+
127	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	M	+	+(3)	+
128	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	M	+	+(3)	+
129	CBA x CBA/H-T6 ⟷ C57BL/6	♀	10/12/75	M	+	+(3)	+
130	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	S	+	+(3)	+
131	CBA x CBA/H-T6 ⟷ C57BL/6	♂	10/12/75	S	+	+(3)	+

Hemoglobin[c] Analysis	Chimeric Drift[d]		Spleen-Thymus[e] Discordance	Cryogenic Storage[f]		Chimeric Serum Blocking Experiments
	PWBC	Hb		SWBC	Thymocytes	
-	-	-	-	+	+	+
-	-	-	-	+	+	+
+(2)	+	+	-	+	+	-
-	-	-	-	+	+	+
-	-	-	-	+	+	+
+(2)	+	+	-	+	+	+
-	-	-	-	+	+	+
-	-	-	-	+	+	+
-	-	-	-	+	+	+
-	-	-	-	+	+	+
-	-	-	-	+	+	+
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	+
+(2)	+	+	-	+	+	+
+(2)	+	+	-	+	+	-
+(2)	+	+	-	+	+	-
+(4)	+	+	-	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	-
+(7)	+	+	+	+	+	-

Table 6. (continued)

Mouse Number	Parental Combination	Sex	Birth Date	Coat Color	Cytotoxicity Testing[b] SWBC	PWBC	TWBC
132	C57BL/6 ⟷ A x SJL	♀	10/21/75	S	-	+(2)	-
133	C57BL/6 ⟷ A x SJL	♂	10/21/75	S	+	+(3)	+
134	C57BL/6 ⟷ A x SJL	♀	10/21/75	S	+	+(3)	+
135	C57BL/6 ⟷ A x SJL	♀	10/21/75	S	+	+(3)	+
137	C57BL/6 ⟷ A x SJL	♀	12/22/75	S	+	+(3)	+
138	C57BL/6 ⟷ A x SJL	♀	12/22/75	M	+	+(3)	+
139	C57BL/6 ⟷ A x SJL	♂	12/22/75	M	+	+(3)	+
140	C57BL/6 ⟷ A x SJL	♀	12/22/75	M	+	+(3)	+
141	C57BL/6 ⟷ A x SJL	♀	12/22/75	M	+	+(3)	+
142	C57BL/6 ⟷ A x SJL	♂	12/22/75	M	+	+(3)	+
143	C57BL/6 ⟷ A x SJL	♂	12/23/75	M	+	+(3)	+
144	CBA x CBA/H-T6 ⟷ DBA/1	♂	12/23/75	M	+	+(2)	+
145	CBA x CBA/H-T6 ⟷ DBA/1	♂	12/23/75	S	+	+(2)	+
146	CBA x CBA/H-T6 ⟷ DBA/1	♂	12/23/75	S	+	+(2)	+
148	C57BL/6 ⟷ A x SJL	♂	2/24/76	S	+	+(2)	+
149	C57BL/6 ⟷ A x SJL	♀	2/24/76	S	+	+(2)	+
150	C57BL/6 ⟷ A x SJL	♀	2/24/75	S	+	+(2)	+
151	C57BL/6 ⟷ A x SJL	♂	2/24/75	S	+	+(2)	+
152	C57BL/6 ⟷ A x SJL	♀	2/24/75	M	+	+(2)	+
153	C57BL/6 ⟷ A x SJL	♀	2/24/75	M	+	+(2)	+
154	CBA x CBA/H-T6 ⟷ DBA/1	♂	3/ 9/76	M	+	+(2)	+
157	C57BL/6 ⟷ A	♂	4/11/76	M	+	+(4)	+
158	C57BL/6 ⟷ A	♀	4/11/76	M	+	+(4)	+
159	C57BL/6 ⟷ A	♀	4/11/76	M	+	+(4)	+
160	C57BL/6 ⟷ A	♂	4/19/76	S	+	+(4)	+
162	C57BL/6 ⟷ A	♂	4/19/76	M	+	+(4)	+
163	CBA x CBA/H-T6 ⟷ DBA/1	♀	4/26/76	S	+	+(2)	+
164	CBA x CBA/H-T6 ⟷ DBA/1	♀	4/26/76	M	+	+(2)	+
165	CBA x CBA/H-T6 ⟷ DBA/1	♂	4/26/76	M	+	+(2)	+
166	CBA x CBA/H-T6 ⟷ DBA/1	♂	4/26/76	M	+	+(2)	+
167	C57BL/6 ⟷ A	♂	4/26/76	S	+	+(4)	+
168	C57BL/6 ⟷ A	♂	4/26/76	M	+	+(4)	+
169	C57BL/6 ⟷ A	♂	4/26/76	M	-	+(4)	-
170	C57BL/6 ⟷ A	♀	5/ 9/76	S	+	+(4)	+
171	C57BL/6 ⟷ A	♂	5/ 9/76	M	+	+(4)	+

Hemoglobin[c] Analysis	Chimeric Drift[d] PWBC	Hb	Spleen-Thymus[e] Discordance	Cryogenic Storage[f] SWBC	Thymocytes	Chimeric Serum Blocking Experiments
+(6)	+	+	-	-	-	-
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
+(7)	+	+	+	+	+	+
-	+	-	+	-	-	+
-	+	-	+	-	-	-
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	-
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+
-	+	-	+	-	-	+
-	+	-	+	-	-	-
-	+	-	+	-	-	-
-	+	-	+	-	-	+
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+
+(3)	+	+	-	-	-	-
+(3)	+	+	+	-	-	+
+(3)	+	+	+	-	-	+

Table 6. (continued)

Mouse Number	Parental Combination	Sex	Birth Date	Coat Color	Cytotoxicity Testing[b] SWBC	PWBC	TWBC
172	C57BL/6 ⟷ A	♀	5/ 9/76	M	+	+(4)	+
173	C57BL/6 ⟷ A	♂	5/ 9/76	M	+	+(4)	+
174	CBA x CBA/H-T6 ⟷ DBA/1	♂	5/ 9/76	S	+	+	+
175	CBA x CBA/H-T6 ⟷ DBA/1	♀	5/ 9/76	M	+	+	+
177	CBA x CBA/H-T6 ⟷ A	♂	5/11/76	M	+	+	-
178	CBA x CBA/H-T6 ⟷ A	♂	5/11/76	M	+	+	-
179	CBA x CBA/H-T6 ⟷ A	♂	5/11/76	S	+	+	-
180	CBA x CBA/H-T6 ⟷ A	♂	5/11/76	S	+	+	-
181	CBA x CBA/H-T6 ⟷ A	♀	5/11/76	S	+	+	-
182	DBA/1 ⟷ A	♂	5/11/76	M	+	+	+
183	DBA/1 ⟷ A	♂	5/11/76	S	+	+	+
184	CBA x CBA/H-T6 ⟷ DBA/1	♂	5/24/76	M	+	+	+
186	DBA/1 ⟷ A	♂	5/24/76	S	+	+	+
187	C57BL/6 ⟷ A	♀	5/24/76	S	+	+(4)	+
188	C57BL/6 ⟷ A	♀	5/24/76	S	-	+(4)	-
191	C57BL/10Sn ⟷ A	♀	5/24/76	M	+	+(4)	+
205	C57BL/10Sn ⟷ A	♂	10/18/77	S	-	+(6)	-
206	C57BL/10Sn ⟷ A	♂	10/18/77	S	-	+(6)	-
207	C57BL/10Sn ⟷ A	♂	10/18/77	S	-	+(6)	-
210	C57BL/10Sn ⟷ A	♀	11/14/77	S	-	+(6)	-
211	C57BL/10Sn ⟷ A	♀	11/14/77	S	-	+(6)	-
212	C57BL/10Sn ⟷ A	♂	11/14/77	M	-	+(6)	-
213	C57BL/10Sn ⟷ A	♂	11/14/77	M	-	+(6)	-
214	C57BL/10Sn ⟷ A	♂	11/14/77	M	-	+(6)	-
215	C57BL/10Sn ⟷ A	♂	11/14/77	M	-	+(6)	-

Hemoglobin[c] Analysis	Chimeric Drift[d] PWBC	Hb	Spleen-Thymus[e] Discordance	Cryogenic Storage[f] SWBC	Thymocytes	Chimeric Serum Blocking Experiments
+(3)	+	+	+	-	-	-
+(3)	+	+	+	-	-	+
-	-	-	+	-	-	-
-	-	-	+	-	-	+
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	+	-	-	-
-	-	-	+	-	-	-
-	-	-	+	-	-	-
+(3)	+	+	+	-	-	+
+(3)	+	+	-	-	-	+
+(3)	+	+	+	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-
+(6)	+	+	-	-	-	-

B. Evaluation of the Trypan Blue Dye Exclusion Assay

1. Analysis of anti-spleen sera

The antisera produced against spleen cells were found to show equal killing of spleen and peripheral white blood cells. This was to be expected since H-2 antigens are strongly expressed on both of these types of cells. In all cases, the killing of cells of the strain in which each antiserum sample was produced was less than 10% (Figure 9).

As expected, the spleen antisera showed poor killing of thymocytes, since 80-90% of thymocytes show only a low concentration of H-2 antigens (Klein, 1975). The errors in the analysis of peripheral white blood cells and spleen white blood cells were determined by analyzing artificial mixtures of cells. As shown in Figure 9, anti-C57BL/6 prepared in A mice, gave the predicted killing of artificial mixtures of C57BL/6 and A peripheral white blood cells, with no significant killing of A target cells. Based on many repeated determinations, the maximum error in the spleen white blood cell assays is estimated to be $\pm$ 10%, while the error in the peripheral white blood cell data is $\pm$ 20%. Depending upon the particular preparation being tested and the strength of the antiserum used, many errors were lower than the maximum value. No antiserum was used if it killed fewer than 50% of the target cells at a 1:2 dilution.

2. Analysis of anti-thymocyte sera

Unlike the spleen antisera, in which cross reactivity was no problem, some of the anti-thymocyte sera showed a high degree of cross reactivity

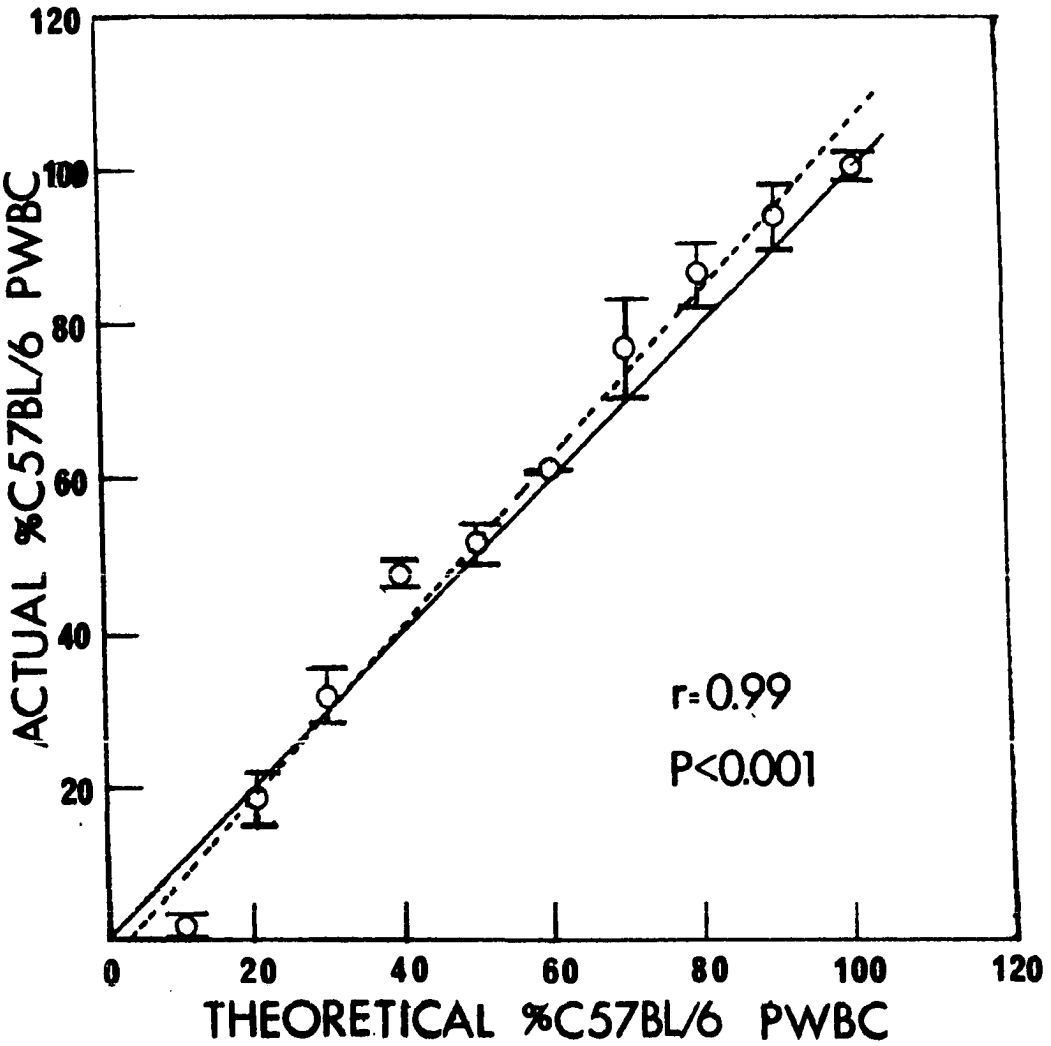


Figure 9. A plot of the percentage of killing of known mixtures of C57BL/6 and A peripheral white blood cells (PWBC) with anti-C57BL/6 serum. [(——) is the theoretical line through the points for a perfect correlation; (---) is the least squares line through the points.]

with the recipient strain. This wasprobably due to contamination of some of the serum samples. Fortunately, not all anti-thymocyte sera cross-reacted and the composition of at least one parental type of thymocytes in allophenic mice or artificial mixtures could be tested without the cross reactivity problem. Based on repeated determinations of artificial mixtures, the maximum error in the thymocyte determinations is estimated to be $\pm$ 10%.

C. RNA Synthesis Assay

1. Analysis of antisera

Five antiserum samples obtained either from the National Institutes of Health or from whole spleen cell immunizations according to the method of Batchelor (1973) were initially titered against spleen cell suspensions using the trypan blue dye exclusion assay.

Antiserum to CBA spleen cells was prepared by immunization of C57BL/6 mice. Reciprocal immunizations were used to produce antisera against C57BL/6 and A spleen cells, to eliminate cross reaction in cell mixing experiments. Thus, C57BL/6 mice were injected with A spleens, and A mice were injected with C57BL/6 spleens. Anti-H-2 sera for use on the DBA/1 mice (anti-$H\text{-}2^q$) and on the SJL mice (anti-$H\text{-}2^s$) were obtained from Research Resources Branch, National Institutes of Health.

All antisera were titered using a constant number of target cells (12×10^4 spleen white blood cells) in each assay tube. At a 1:16 anti-serum dilution, it was found that four of the antisera killed $\geq$ 95% of the

correct target cells with less than 10% killing of control cells. The anti-H-2^q serum gave killing endpoint ranges of 70-90% in different experiments. None of the serum samples showed anti-complementary effects when used with guinea pig serum as the source of complement. The five antiserum samples were used for all subsequent experiments described in this section.

2. Conditions for RNA synthesis assay

Figure 10 shows the counts per minute (cpm) in 12 x 10^4 spleen cells (A strain) labeled for the five hours with varying amounts of [^{3}H]uridine. Incorporation into high molecular weight product was linear in the ranges 2.5 to 15 μ Ci [^{3}H]uridine/assay. Cells labeled with amounts of [^{3}H]-uridine greater than 20 μ Ci showed decreased counts incorporated. A concentration of 12.5 μ Ci [^{3}H]uridine/assay was chosen for all subsequent experiments.

The optimal labeling period for spleen cells was five hours, as is shown in Figure 11. The incorporation of label into total RNA was linear for the five-hour period. Longer periods of labeling were found to be toxic to the cells. Thus, a five-hour labeling period was used for all subsequent experiments.

Figures 12a, 12b, 12c, 12d, and 12e represent counts incorporated into spleen cells following different times of preincubation with either appropriate antiserum or normal serum and complement. Cells were labeled for a five-hour period following the preincubation. In all five experiments, no detectable RNA synthesis was observed after a one hour preincubation

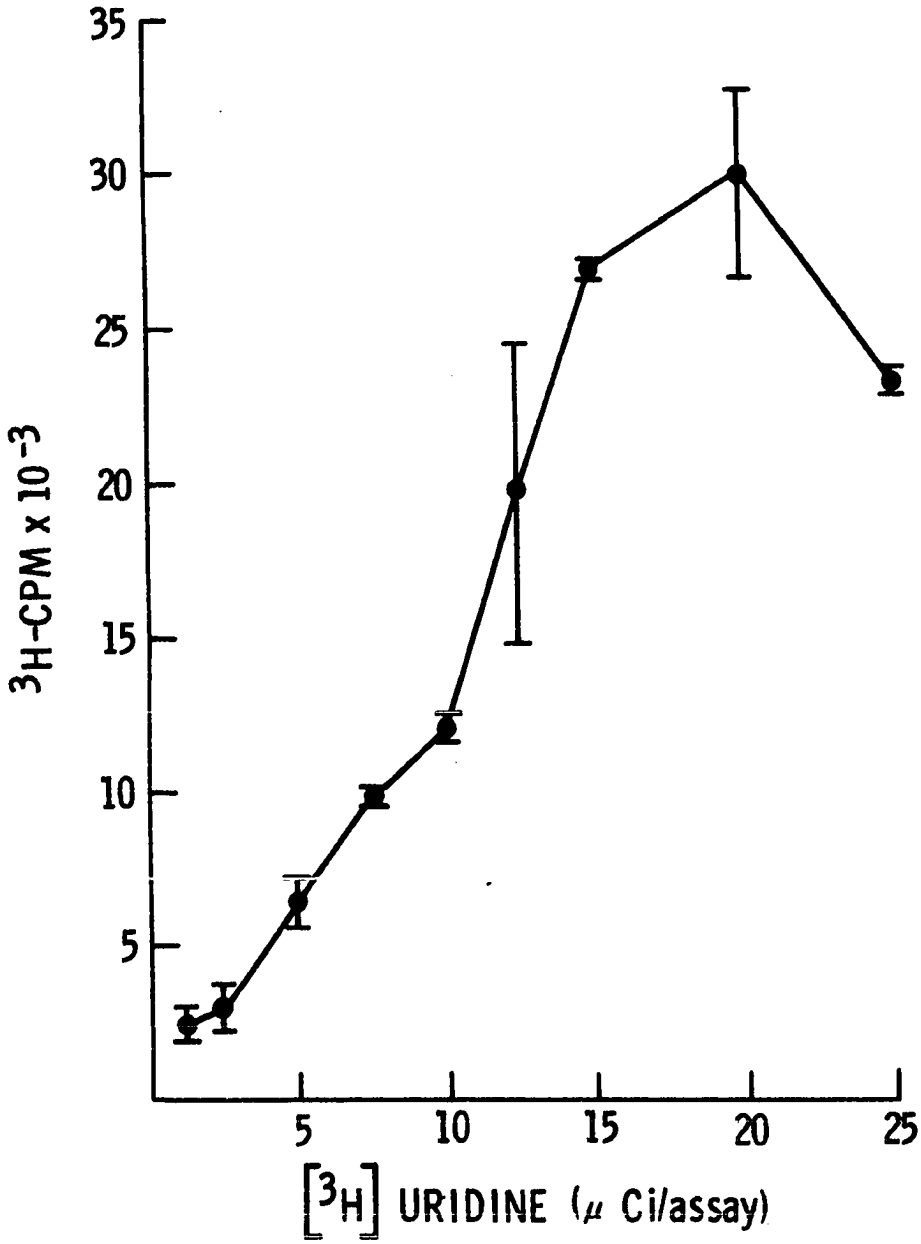


Figure 10. Optimal concentration of [^{3}H]uridine. [Counts per minute incorporated into high molecular weight product as a function of concentration of [^{3}H]uridine.]

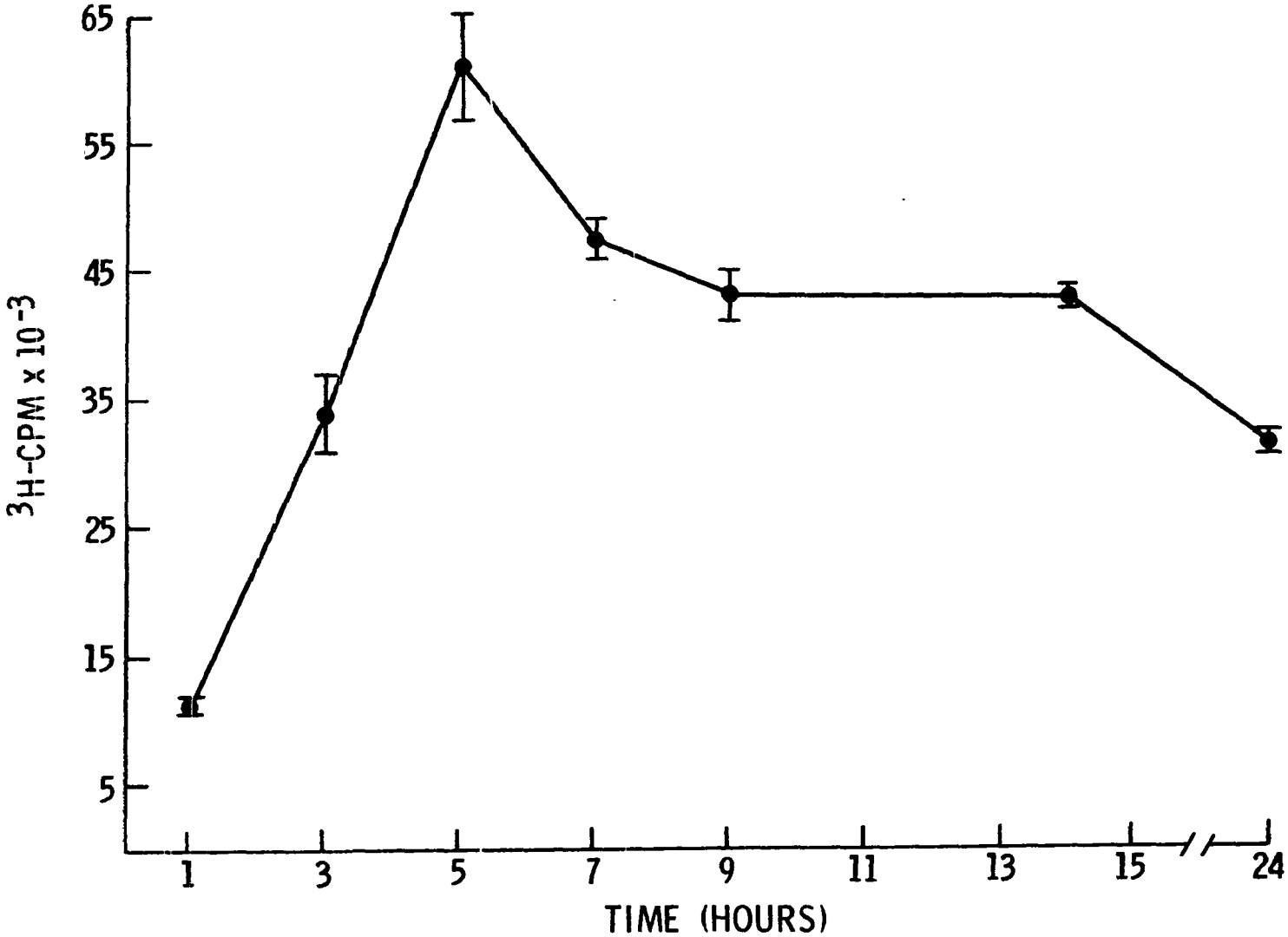


Figure 11. Optimal time of labeling. [Counts per minute incorporated into high molecular weight product as a function of time of labeling in the presence of [^{3}H]uridine.]

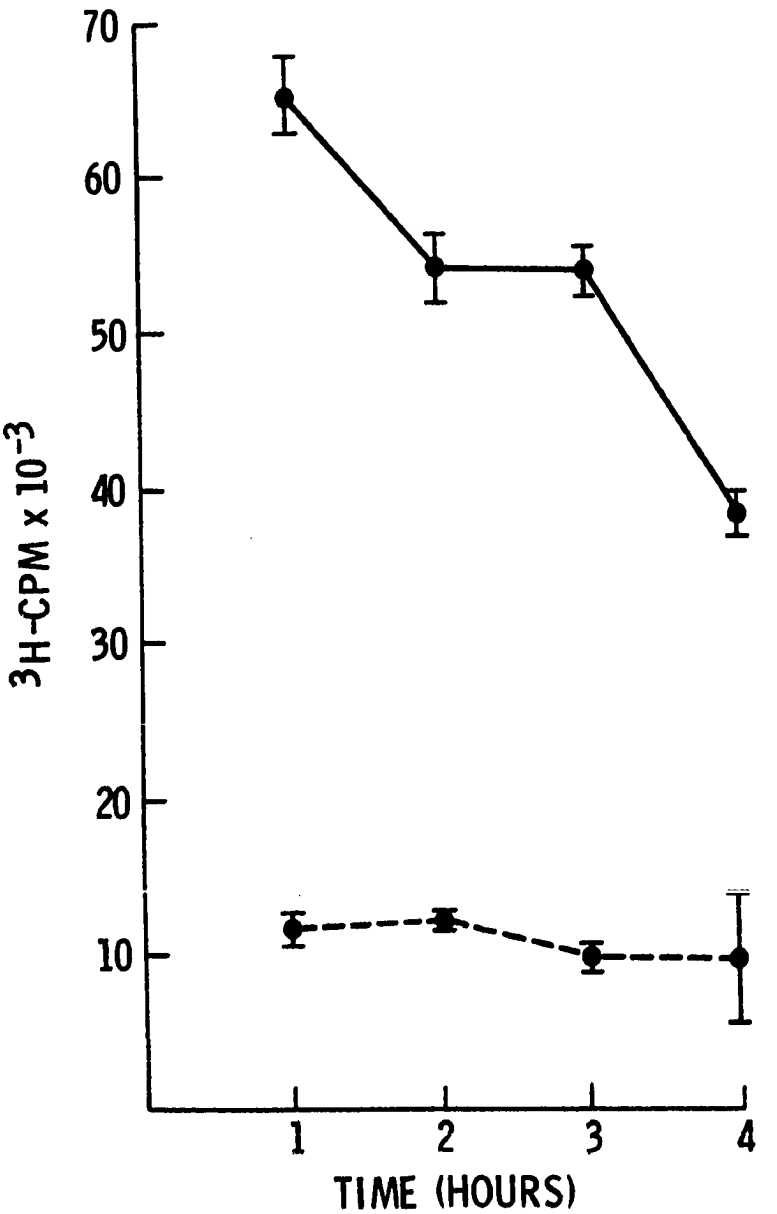
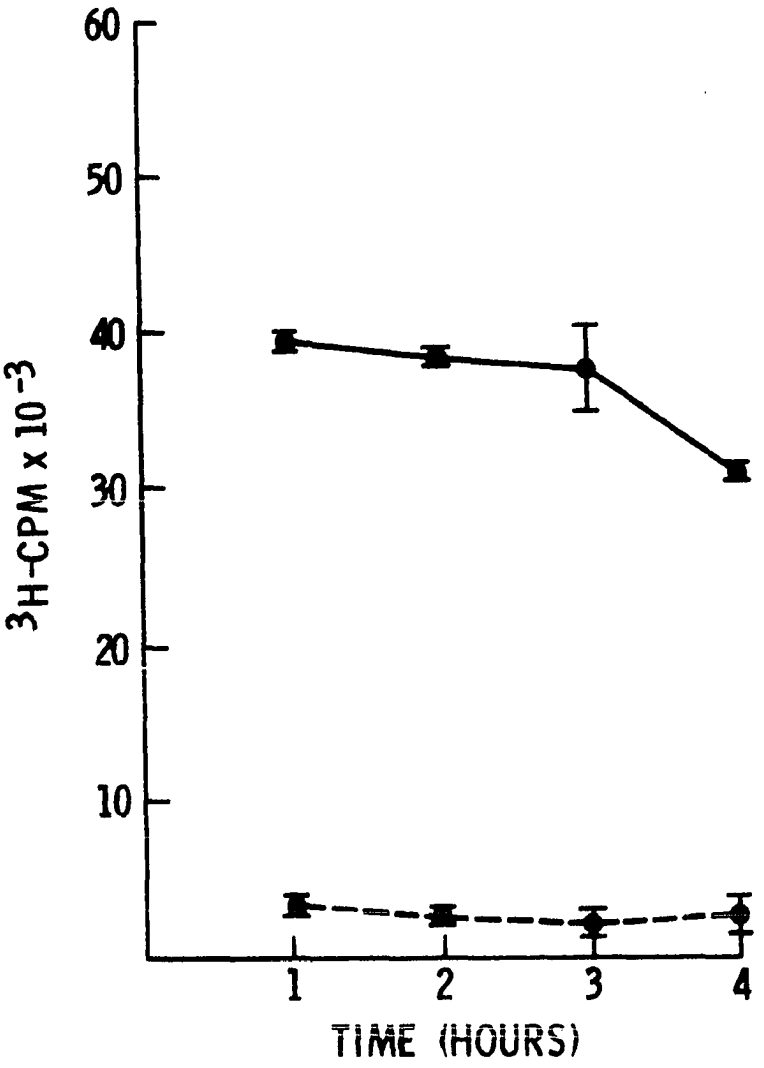


Figure 12a. Optimal time of incubation. [Counts per minute incorporated into high molecular weight product by spleen cells, incubated five hours in the presence of 12.5 μCi [^{3}H]uridine per assay, following different periods of preincubation with antiserum and complement. Cells treated with a 1:16 dilution of normal mouse serum (——); cells treated with a 1:16 dilution of antiserum (---).] (A spleen cells and anti-A serum)



12b. C57BL/6 spleen cells and anti-C57BL/6 serum

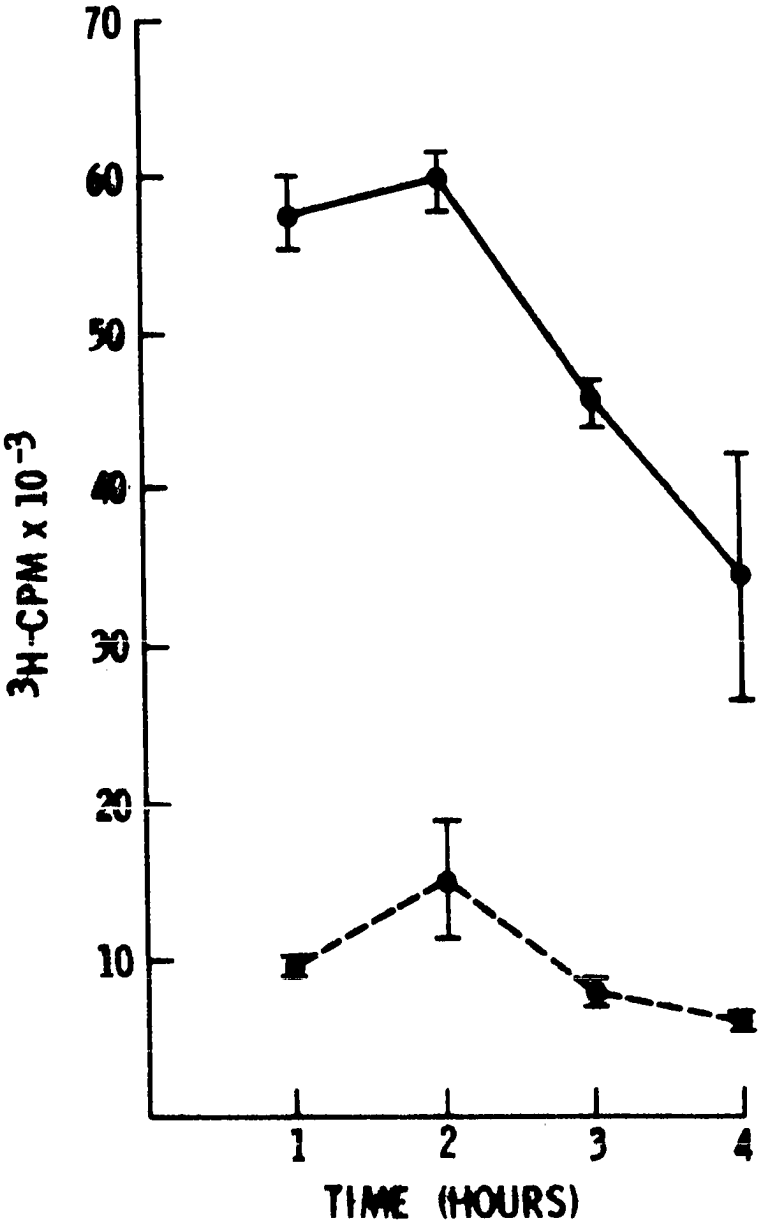


Figure 12c. CBA spleen cells and anti-CBA serum

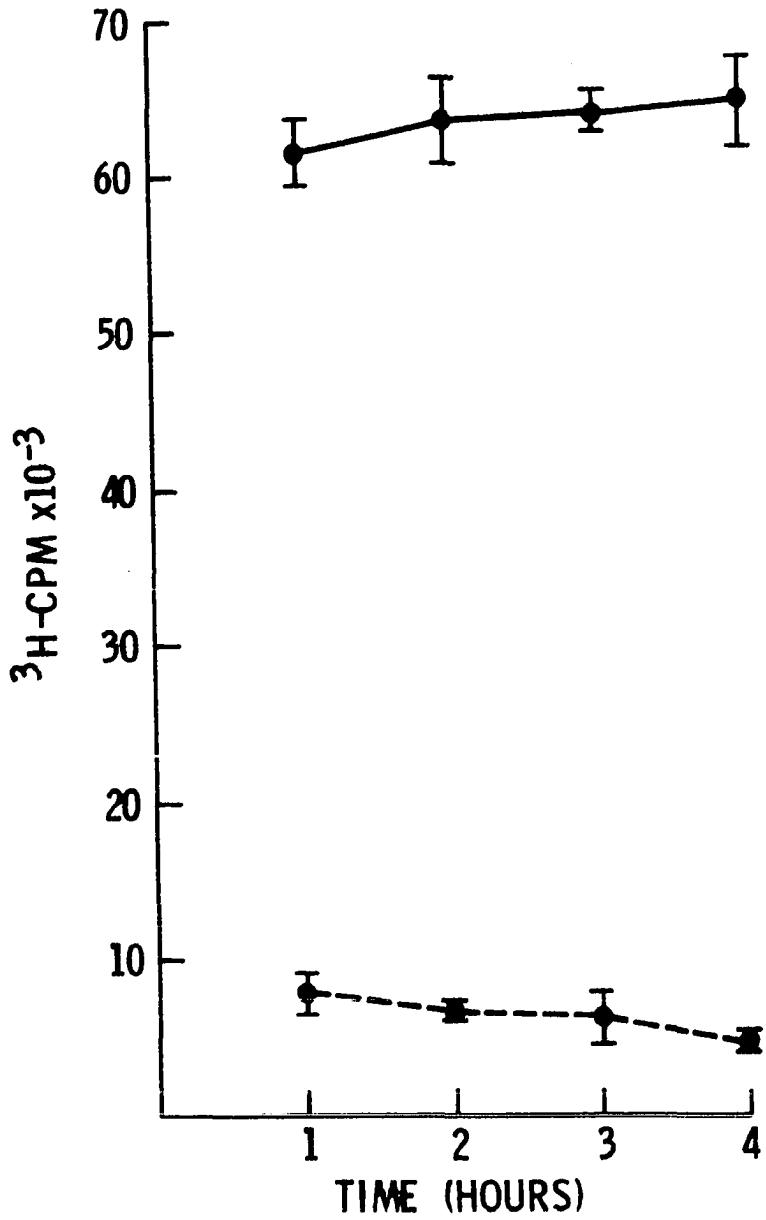


Figure 12d. DBA/1 spleen cells and anti-DBA/1 serum

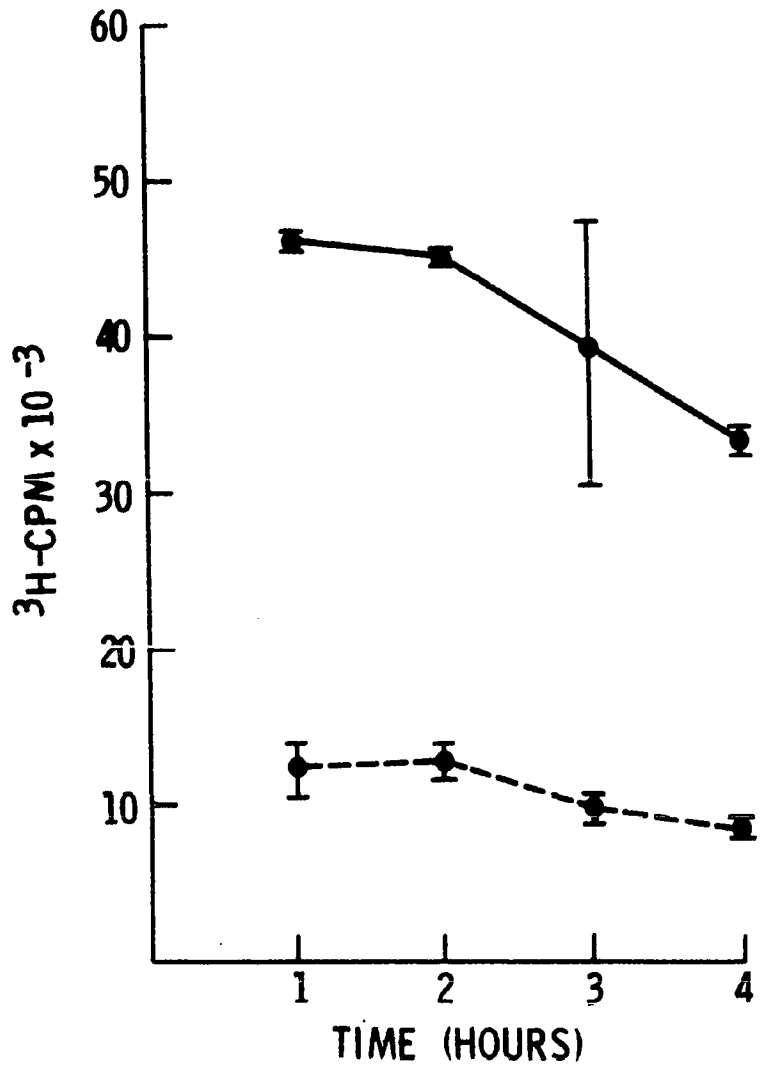


Figure 12e. SJL spleen cells and anti-SJL serum

of cells with antiserum and complement. Samples treated with normal serum and complement showed good viability for a two-hour period, after which counts decreased. This is to be expected since guinea pig serum is known to contain some cytolysins (Sanderson, 1964). This could potentially be overcome by substituting rabbit serum as the source of complement. Thus, a one-hour preincubation period, and a five-hour labeling period with 12.5 μ Ci/assay [^{3}H]uridine were the final conditions chosen for the RNA synthesis assay.

3. Serum dilution experiments

Figures 13a, 13b, 13c, 13d, and 13e demonstrate comparative changes in cytotoxicity in both the trypan blue dye exclusion and RNA synthesis assays as a result of increasing serum dilutions. The target cell number (12×10^{4} spleen white blood cells/assay) remained constant in these experiments. The curves for all five strains were subjected to a computerized probit analysis (Smith et al., 1977) to determine the 50% endpoint for both the RNA synthesis assay and the trypan blue dye exclusion assay. The 50% cytotoxic endpoints were examined because they represent the most sensitive part of the dilution curve (Brunner et al., 1968). The data in Table 7 clearly show that identical serum titers are obtained using the two different assay procedures.

4. Cell dilution experiments

Figures 14a, 14b, 14c, 14d, and 14e show the results of the RNA synthesis assay on various numbers of spleen cells of the five different mouse

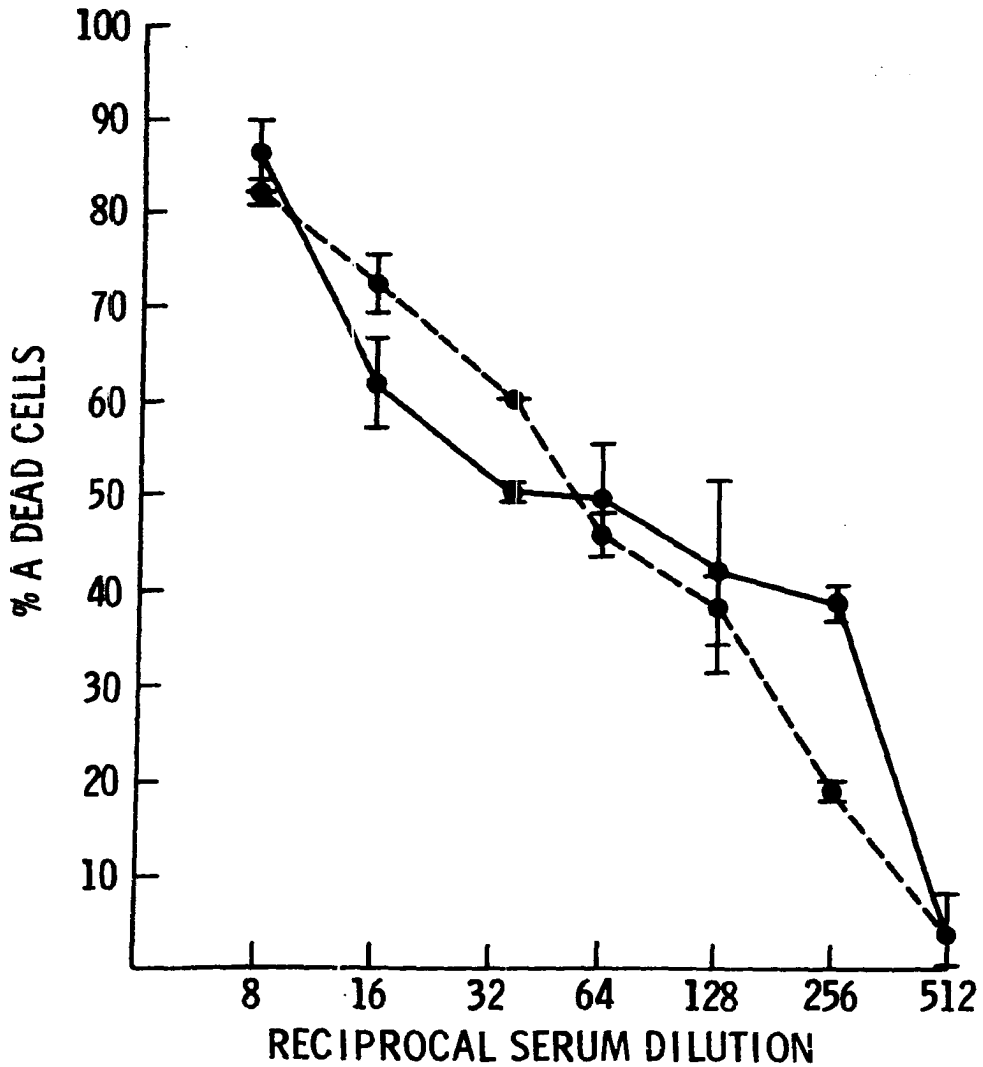


Figure 13a. Analysis of serum titer. [The percentage of dead cells as a function of reciprocal dilutions of antiserum. Each assay was performed as described in the text using spleen cells as the target cells. Data are shown for the RNA synthesis assay (——) and the trypan blue dye exclusion assay (---).] (A spleen cells and anti-A serum)

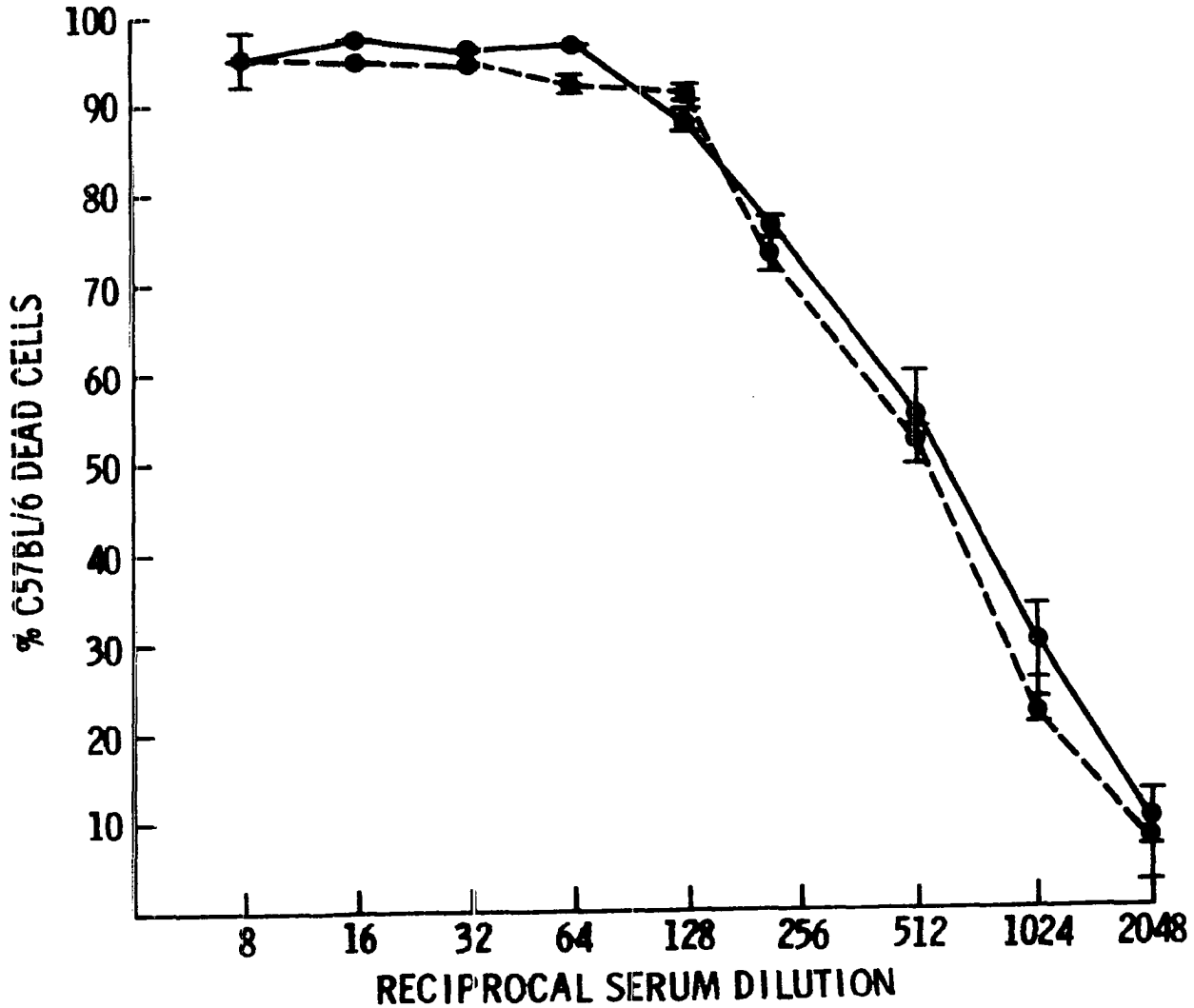


Figure 13b. C57BL/6 spleen cells and anti-C57BL/6 serum

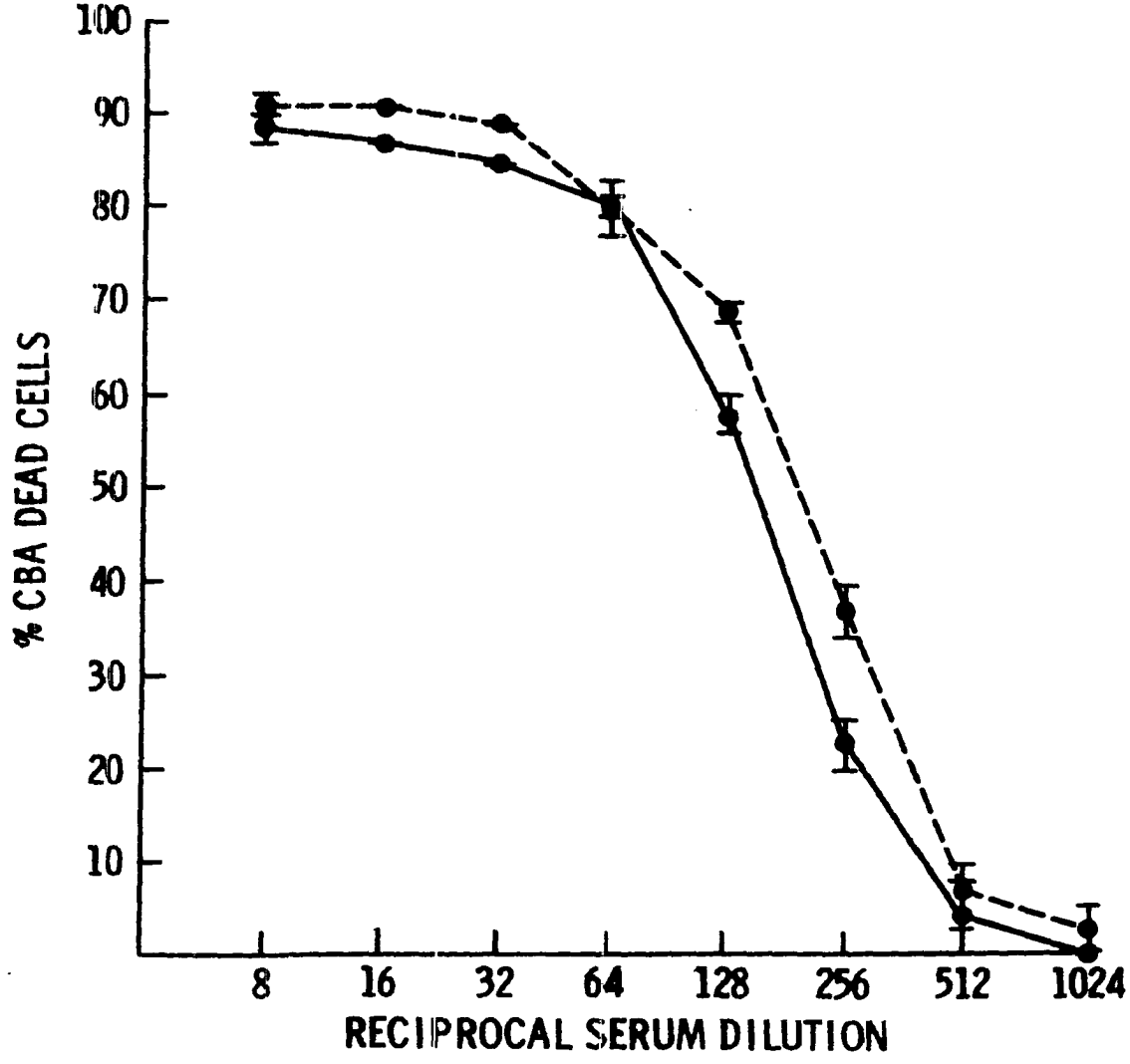


Figure 13c. CBA spleen cells and anti-CBA serum

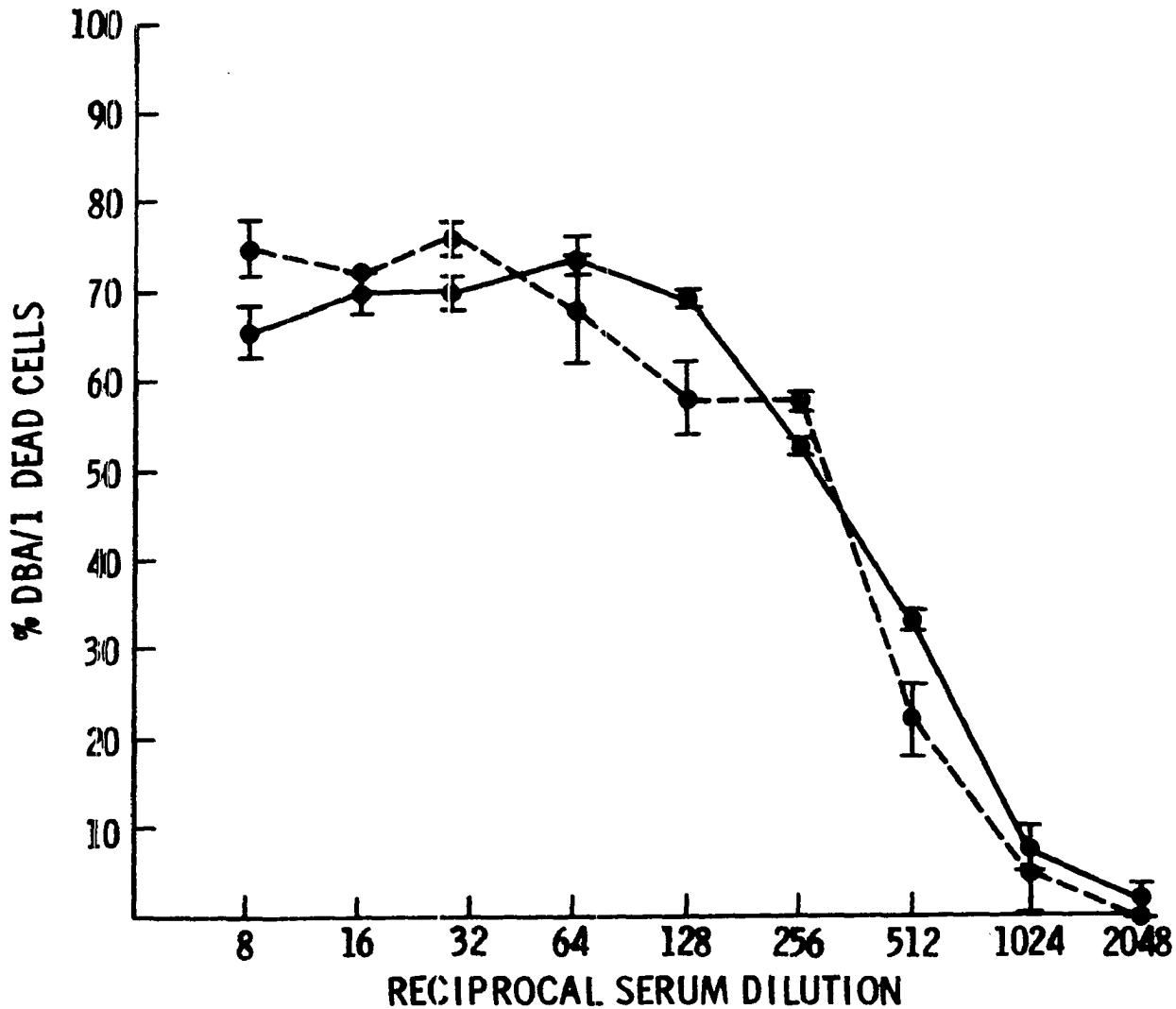


Figure 13d. DBA/1 spleen cells and anti-DBA/1 serum

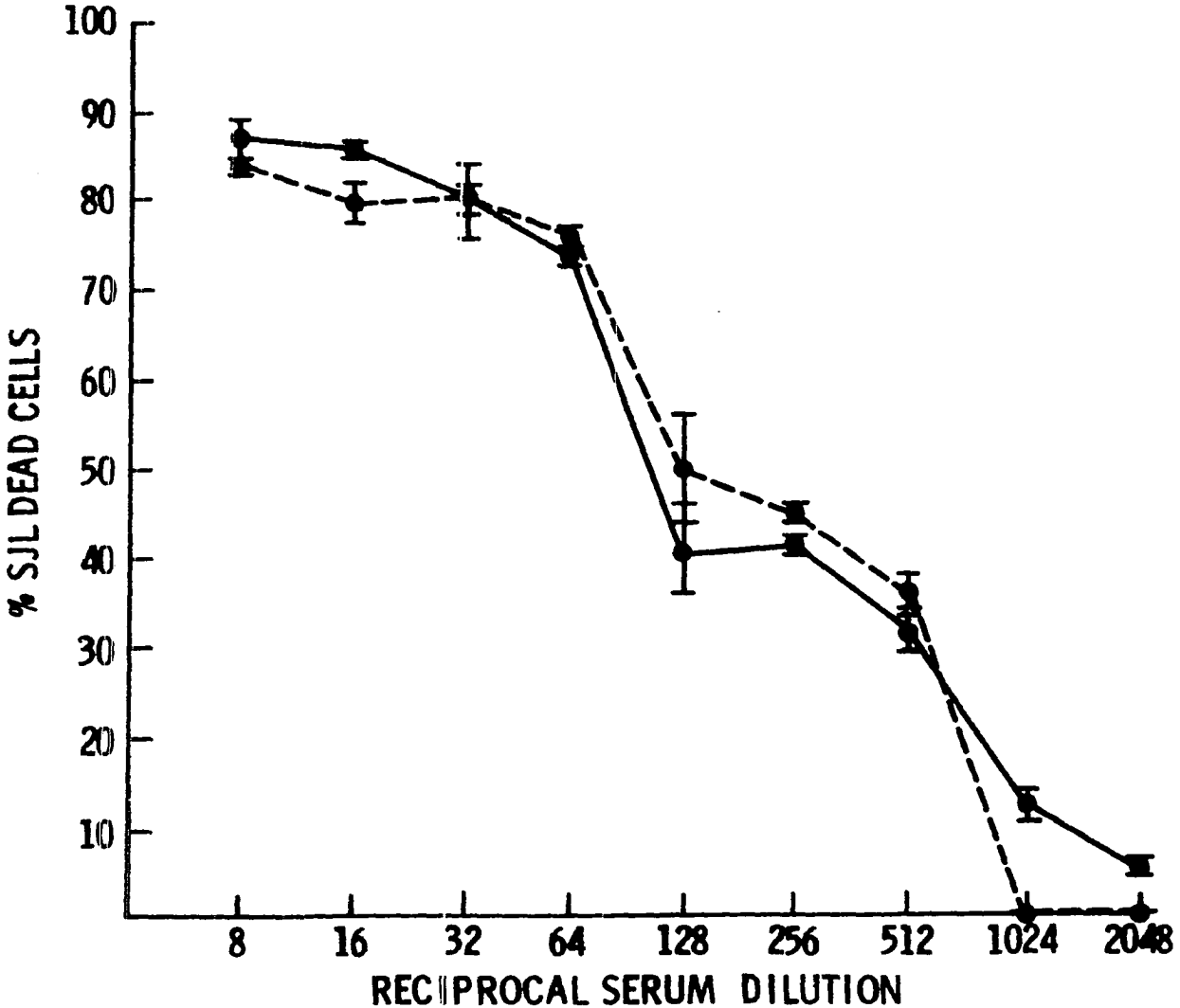


Figure 13e. SJL spleen cells and anti-SJL serum

Table 7. Antiserum titers determined by RNA synthesis assay and by trypan blue dye exclusion assay[a]

Antisera	Titer	
	RNA Synthesis Assay	Trypan Blue Dye Exclusion Assay
Anti-A	71	71
Anti-C57BL/6	537	468
Anti-CBA	100	138
Anti-DBA/1	209	166
Anti-SJL	158	182

[a]Data are the results of a probit analysis of the titration curves according to Smith et al. (1977). The values are the computer calculated reciprocal serum dilutions giving a 50% cytotoxic endpoint.

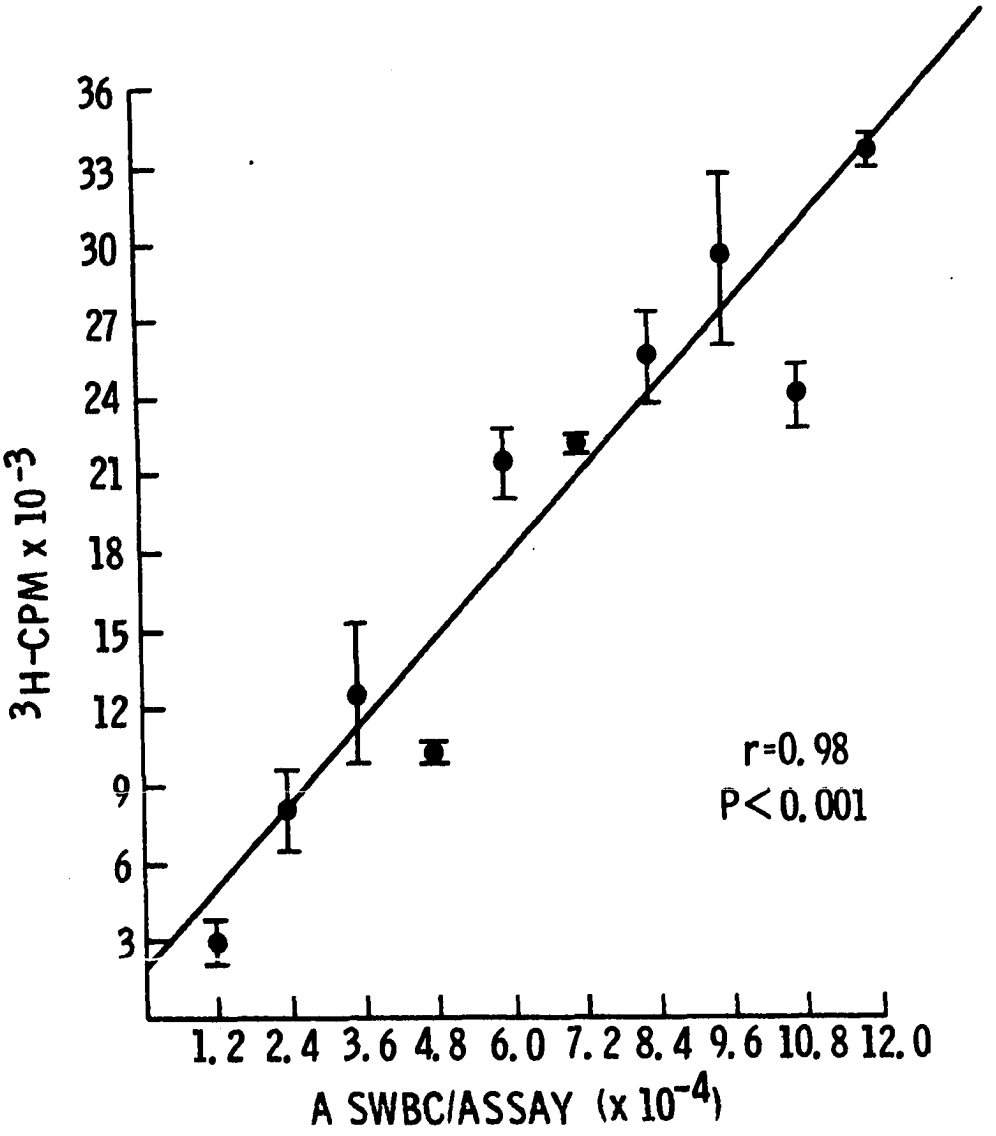


Figure 14a. Analysis of RNA synthesis assay. [The counts per minute incorporated into high molecular weight product as a function of concentration of spleen cells. The least squares linear regression line through the points is shown. The correlation coefficient, r, and probability, P, that the points are not correlated, have been calculated according to Bevington (1969).] (A spleen cells)

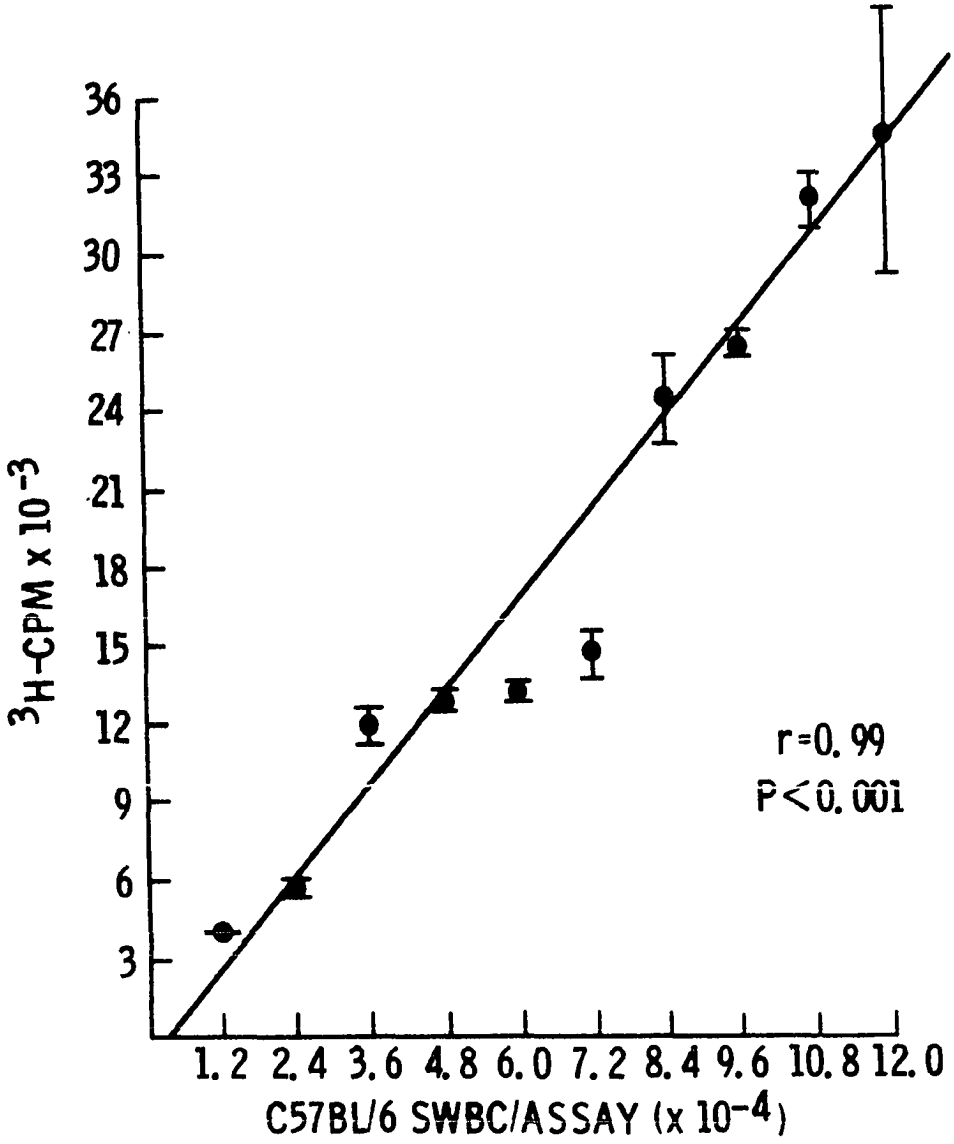


Figure 14b. C57BL/6 spleen cells

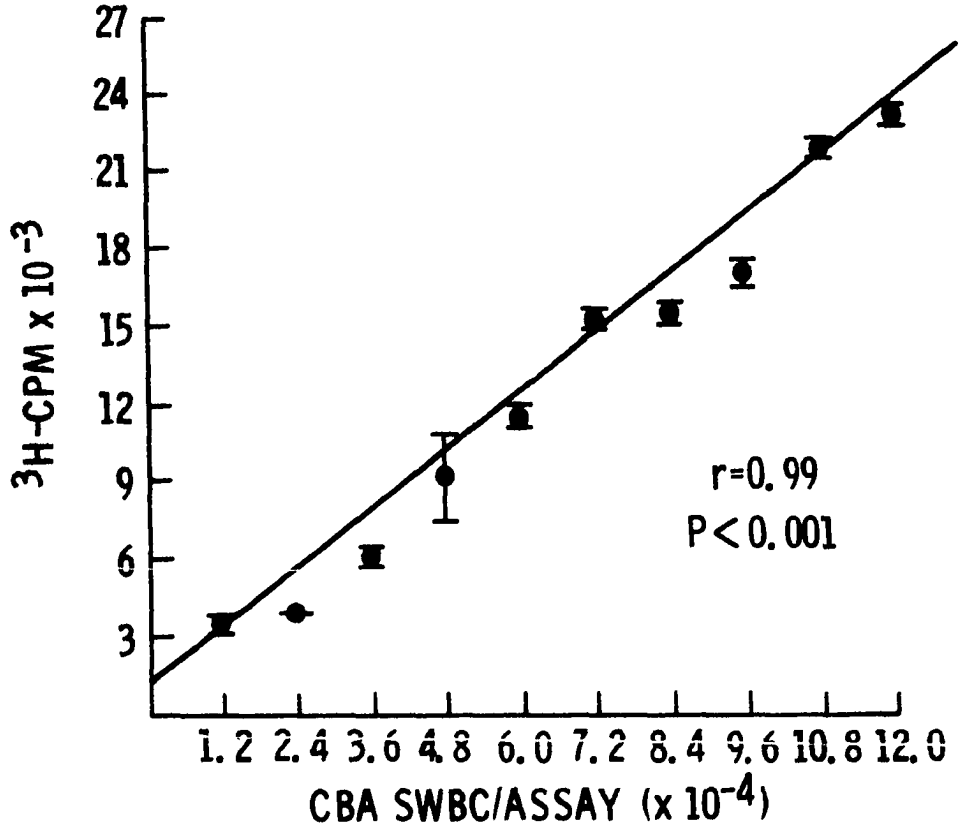


Figure 14c. CBA spleen cells

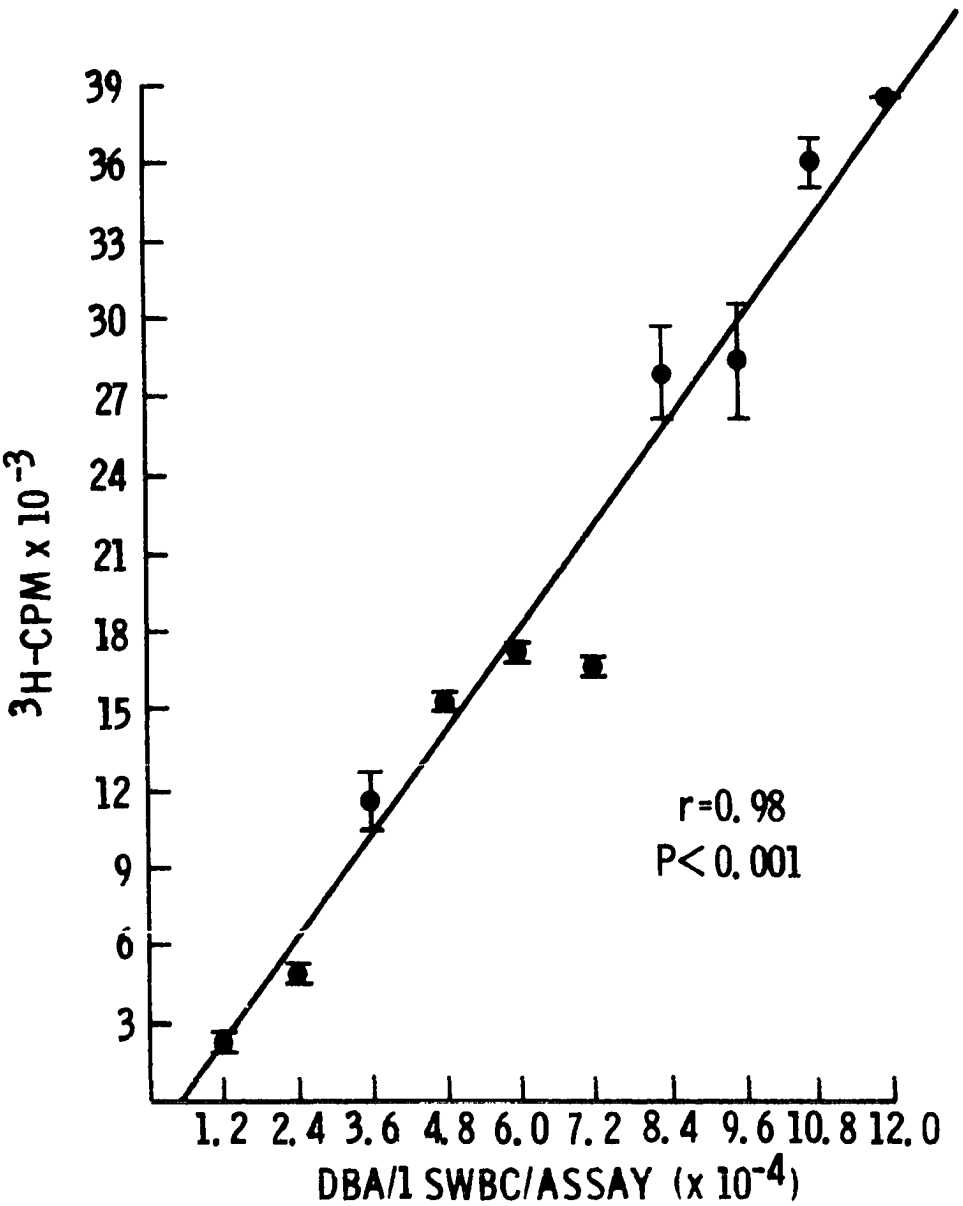


Figure 14d. DBA/1 spleen cells

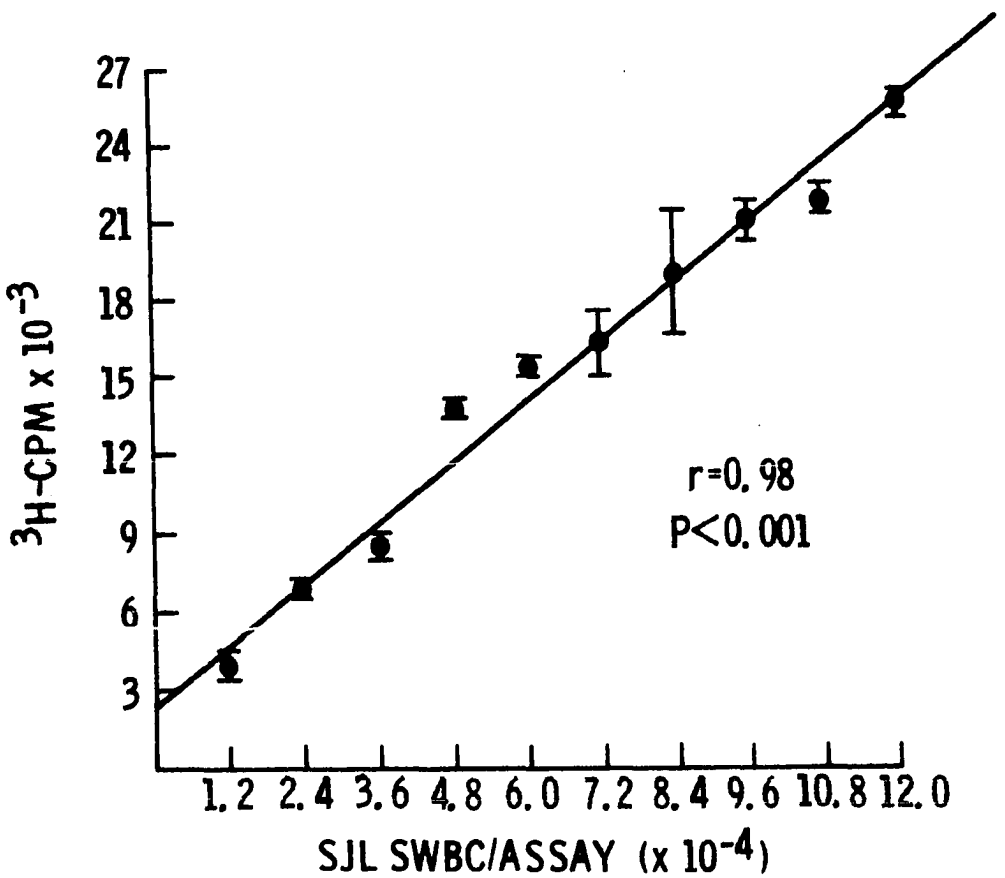


Figure 14e. SJL spleen cells

strains. The cpm incorporated reflect the number of live cells in each sample, so that an unknown sample may be analyzed simultaneously by comparison with the standard curves. Differences observed in counts incorporated by identical numbers of cells from mice of different strains were found to reflect the uniqueness of each individual cell population, rather than to reflect consistent differences among mouse strains. Thus, the differences observed within one strain from day to day were as great as differences observed among strains. For this reason, control experiments must be included every time an assay is performed.

Similar samples were simultaneously evaluated using the trypan blue dye exclusion assay. Figures 15a, 15b, 15c, 15d, and 15e represent the analysis of these samples. As described in Materials and Methods, these samples contained mixtures of two cell types, and were treated with antisera directed to one of the cell types. The percentage of dead cells was a reflection of the total number of cells of that type in the population.

Table 8 shows a comparison of the data from the two assay procedures utilizing all the data collected from duplicate determinations on each of the five mouse strains (Figures 14a, 14b, 14c, 14d, 14e, and 15a, 15b, 15c, 15d, and 15e). The estimated error in both the RNA synthesis and the trypan blue dye exclusion assays is less than 10%. As can be observed, the RNA synthesis assay gives comparable or better accuracy than the trypan blue dye exclusion assay.

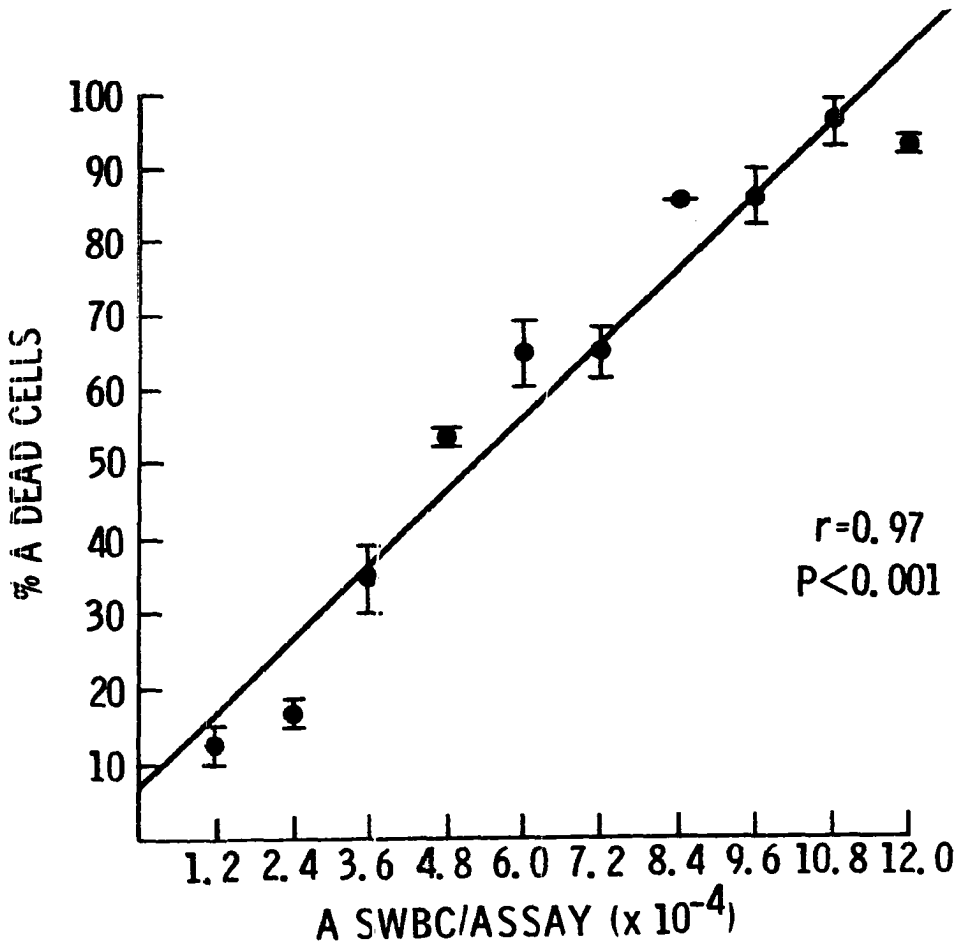


Figure 15a. Analysis of trypan blue dye exclusion assay. [The percentage of dead cells as a function of concentration of spleen cells of a particular strain. The least squares linear regression line through the points is shown. The correlation coefficient, r, and probability, P, that the points are not correlated, have been calculated according to Bevington (1969).] (A spleen cells mixed with C57BL/6 cells, anti-A serum)

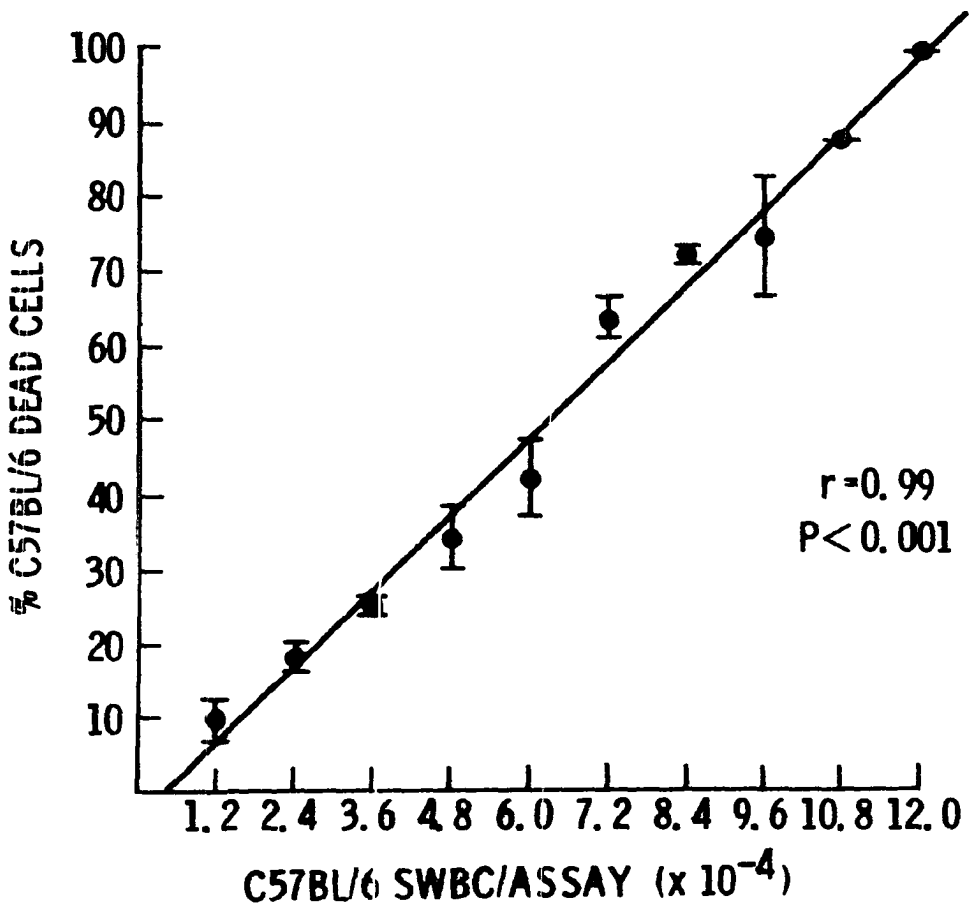


Figure 15b. C57BL/6 spleen cells (mixed with A cells), anti-C57BL/6 serum

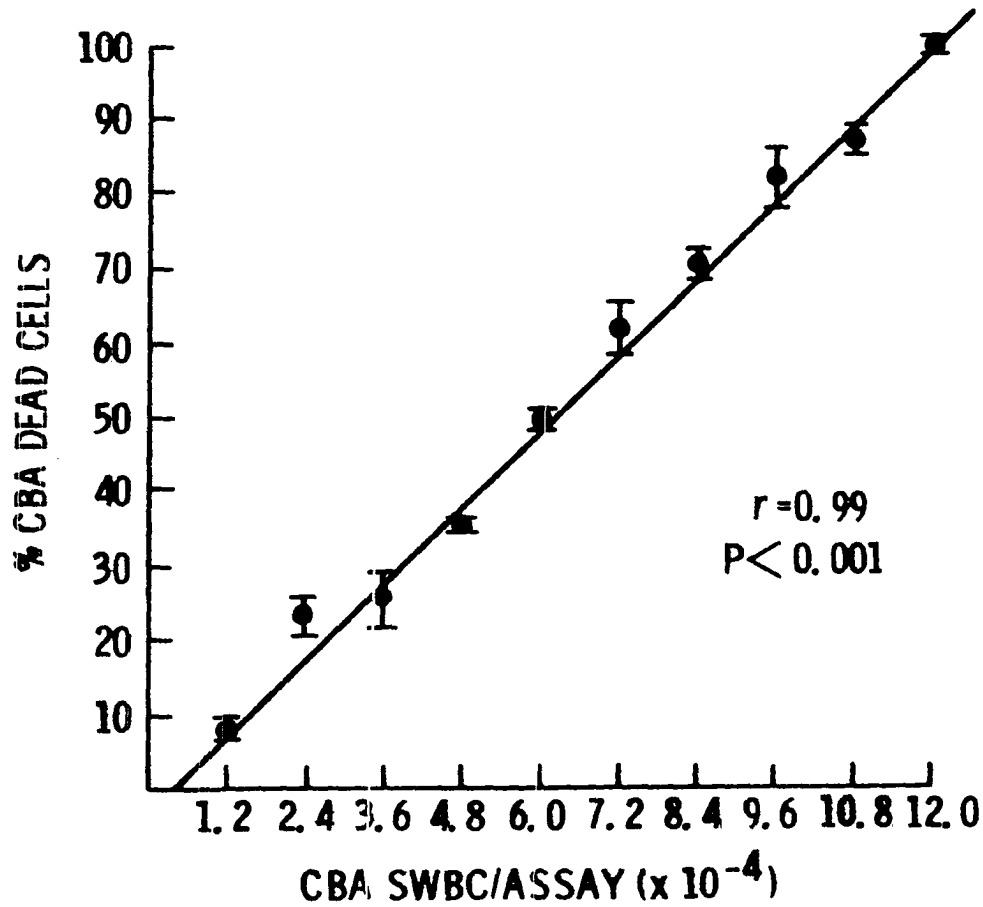


Figure 15c. CBA spleen cells (mixed with C57BL/6 cells), anti-CBA serum

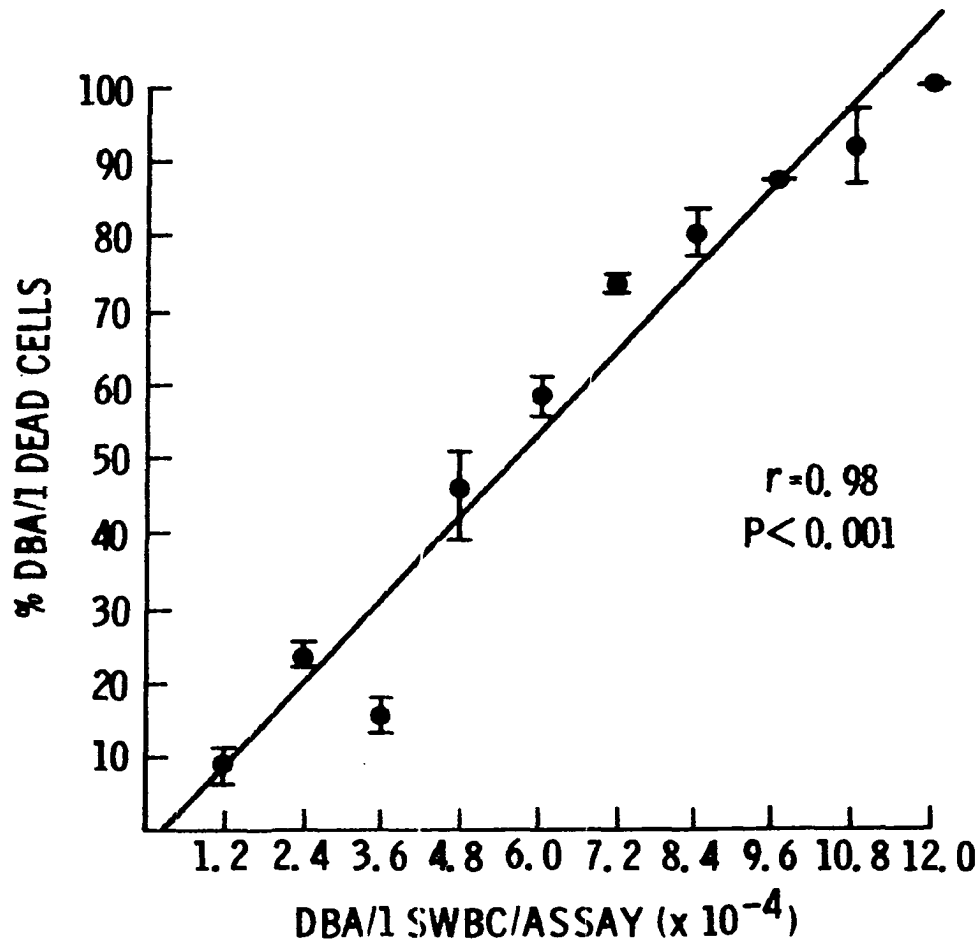


Figure 15d. DBA/1 spleen cells (mixed with C57BL/6 cells), anti-DBA/1 serum

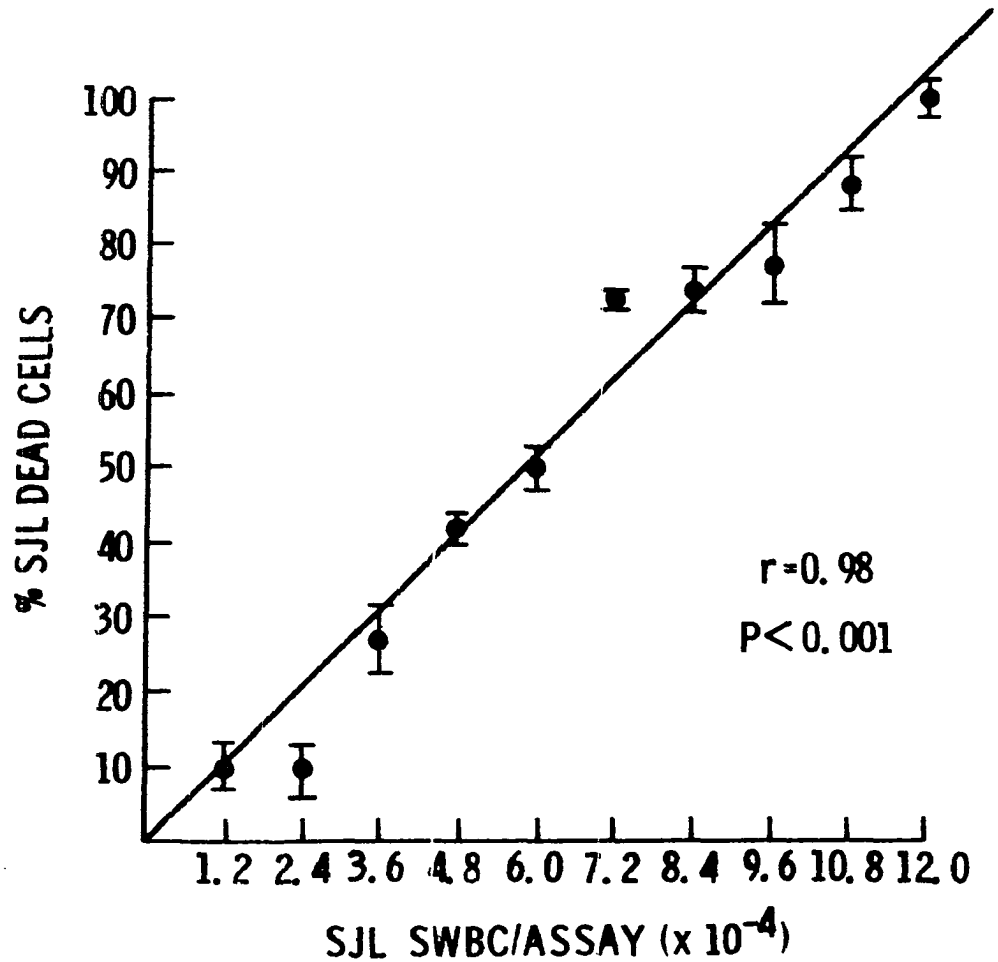


Figure 15e. SJL spleen cells (mixed with C57BL/6 cells), anti-SJL serum

Table 8. Comparison of RNA synthesis assay and trypan blue dye exclusion assay[a]

Theoretical Cell Number x 10^4	Number Determinations	Experimental Cell Number x 10^4	
		RNA Synthesis Assay	Trypan Blue Dye Exclusion Assay
12.0	10	12.1 ± 0.8	11.7 ± 0.6
10.8	10	10.7 ± 1.2	10.7 ± 0.6
9.6	10	9.5 ± 1.1	9.6 ± 0.9
8.4	10	8.6 ± 0.9	9.0 ± 0.7
7.2	10	6.7 ± 1.2	7.9 ± 0.4
6.0	10	6.1 ± 1.0	6.3 ± 1.0
4.8	10	4.7 ± 1.0	5.0 ± 0.9
3.6	10	3.7 ± 0.9	3.1 ± 0.9
2.4	10	2.2 ± 0.5	2.3 ± 0.6
1.2	10	1.2 ± 0.4	2.1 ± 2.7

[a]Data are compiled from duplicate assays of the A, C57BL/6, CBA, DBA/1, and SJL strains.

D. Evaluation of Cryogenic Storage of Lymphocytes

1. The effect of "freeze-thawing" on lymphocyte viability

The possible lethal effects of controlled "freeze-thawing" on the composition of spleen white blood cells and thymus cells from allophenic mice were evaluated by testing for least significant differences prior to, and after, cryogenic storage. Analysis of organ compositions were made at the time of animal sacrifice and again one-two years later. Table 9 summarizes the results from 26 allophenic mice of five combinations. These combinations included 10 C57BL/6 $\longleftrightarrow$ (A x SJL)F_1, 3 DBA/1 $\longleftrightarrow$ (A x SJL)F_1, 6 (CBA x CBA/H-T6)F_1 $\longleftrightarrow$ DBA/1, 4 (CBA x CBA/H-T6)F_1 $\longleftrightarrow$ C57BL/6, and 3 C57BL/6 $\longleftrightarrow$ DBA/1 mice.

Data are shown in Table 10 for the analysis of thymus composition from 21 allophenic mice of similar combinations. All the numbers in Table 9 and 10 are the average of duplicate determinations. The average percentages of nonspecific cell death for spleen white blood cells and thymus cells were 16% and 9%, respectively.

2. The effects of "freeze-thawing" on basal levels of RNA synthesis in lymphocytes

Basal levels of RNA synthesis in normal serum treated, frozen-thawed C57BL/6 and (A x SJL)F_1 spleen white blood cells were examined using the previously described RNA synthesis assays. Viability at the termination of the experiment was evaluated using the trypan blue dye exclusion assay.

Table 9. The effects of freeze-thawing on allophenic mouse spleen white blood cell viability

Mouse Number	% Nonspecific[a] Cell Death	Spleen White Blood Cells[b,c] Freshly Prepared %C57BL/6	Freshly Prepared %A x SJL	Frozen-Thawed %C57BL/6	Frozen-Thawed %A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	16	2	112	1	88
134	5	2	106	7	98
135	12	2	106	1	97
137	22	5	85	1	83
138	18	3	101	0	96
139	17	1	96	0	99
140	10	3	93	15*	91
141	6	11	102	19*	91
142	10	14	86	10	78
143	10	23	61	18	76
		%DBA/1	%A x SJL	%DBA/1	%A x SJL
B. DBA/1 ⟷ (A x SJL)F_1					
94	21	7	--	0	98
99	23	14	--	0*	92
100	24	16	--	1**	90

[a]Nonspecific percent cell death is based on duplicate values determined by the trypan blue dye exclusion assay approximately 4-6 hours after thawing cells.

[b]Freshly prepared spleen white blood cells refers to the spleen composition of allophenic mouse determined at the time of sacrifice by duplicate trypan blue dye exclusion assays.

[c]Significance was determined by testing for the probability of a least significant difference with a Student's "t" test. (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[d]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 9. (continued)

Mouse Number	% Nonspecific[a] Cell Death	Spleen White Blood Cells[b,c] Freshly Prepared %CBA[d]	Freshly Prepared %DBA/1	Frozen-Thawed %CBA[d]	Frozen-Thawed %DBA/1
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	16	33	74	25	80
103	16	89	4	93	8
104	28	70	22	76	26
105	14	54	48	50	46
106	15	2	102	2	96
107	14	16	85	14	81
		%CBA[d]	%C57BL/6	%CBA[d]	%C57BL/6
D. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6					
119	23	18	58	40*	56
120	25	83	16	48**	49**
125	16	15	86	21	67**
127	25	17	69	32	70
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
E. C57BL/6 ⟷ DBA/1					
101	9	14	100	--	90
123	9	69	7	--	9
124	9	42	35	--	18*

Table 10. The effects of freeze-thawing on allophenic mouse thymocyte viability

Mouse Number	% Nonspecific[a] Cell Death	Thymocytes[b,c] Freshly Prepared		Thymocytes[b,c] Frozen-Thawed	
		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	10	--	99	12	94
134	6	--	92	5	87
135	12	--	97	9	84
137	8	--	105	22	93
138	10	--	94	21	81
139	12	--	93	7	85
140	9	--	105	4	94
141	--	--	100	--	--
142	10	--	71	1	94**
143	--	--	64	--	--
		%DBA/1	%A x SJL	%DBA/1	%A x SJL
B. DBA/1 ⟷ (A x SJL)F_1					
94	6	--	--	50	49
99	10	--	--	17	80
100	13	--	--	31	56

[a]Nonspecific percent cell death is based on duplicate values determined by the trypan blue exclusion assay approximately 4-6 hours after thawing cells.

[b]Freshly prepared thymocytes refers to the thymus composition of an allophenic mouse determined at the time of sacrifice by duplicate trypan blue dye exclusion assays.

[c]Significance was determined by testing for the probability of a least significant difference with a Student's "t" test. (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[d]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 10. (continued)

Mouse Number	% Nonspecific[a] Cell Death	Thymocytes[b,c] Freshly Prepared %CBA[d]	Freshly Prepared %DBA/1	Frozen-Thawed %CBA[d]	Frozen-Thawed %DBA/1
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	11	--	--	--	63
103	10	--	--	--	20
105	11	--	--	--	42
106	7	--	--	--	90
107	16	--	--	--	96
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
D. C57BL/6 ⟷ DBA/1					
101	9	--	--	--	105
123	9	--	85	--	84
124	9	--	71	--	81

Results indicated that in samples of spleen cell suspensions showing 70-80% viability, only slightly significant levels of RNA synthesis were measured above background. Control samples containing freshly prepared spleen white blood cells gave counts per minutes four times greater than frozen-thawed cells. Repeated experiments, in which cells were thawed five hours before measuring basal levels of RNA synthesis, gave similar results.

E. Polyacrylamide Gel Isoelectric Focusing (PAGIF)

1. Hemoglobin resolution

Figure 16 shows the isoelectric focusing gels of various inbred strains and F_1 hybrids described in Table 2. These patterns are predicted from the electrophoretic studies of Hutton et al. (1962 a,b) and from the sequencing work of Gilman (1972), which have shown that some inbred strains of mice produce two hemoglobin β-chains in unequal amounts while other strains produce a single β-chain. None of the strains studied have any known electrophoretically distinct products in the hemoglobin α-chain. There are several interesting observations to be made from Figure 16. First, the single type of hemoglobin and diffuse (double) types of hemoglobin migrate to different positions on the isoelectric focusing gels. Second, the two single hemoglobins migrate identically with each other, and the four double hemoglobins migrate identically with each other. Thus, mixtures of two single hemoglobins or two double hemoglobins cannot be resolved. Third, it is seen from the (A x SJL)F_1 mice that the

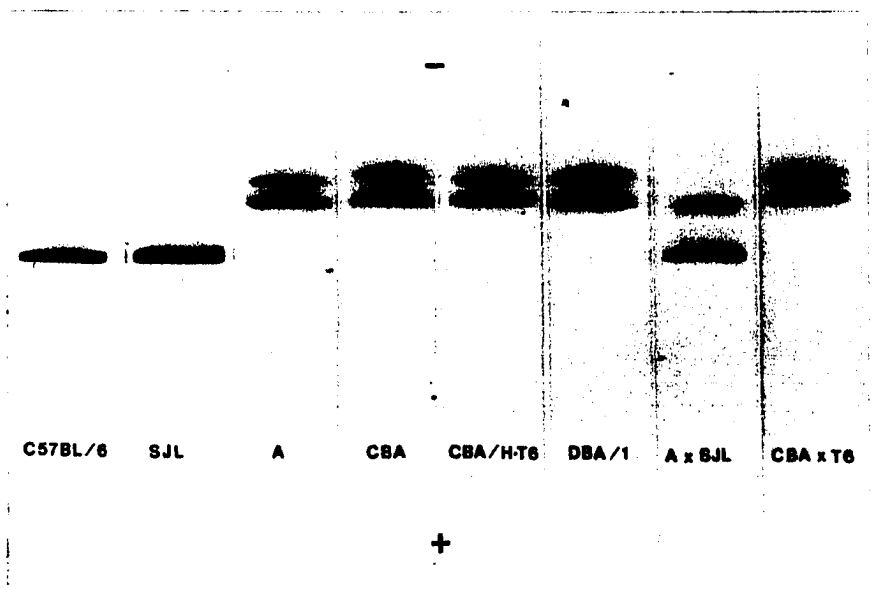


Figure 16. Polyacrylamide gel isoelectric focusing patterns of hemoglobin samples from inbred and F_1 mice

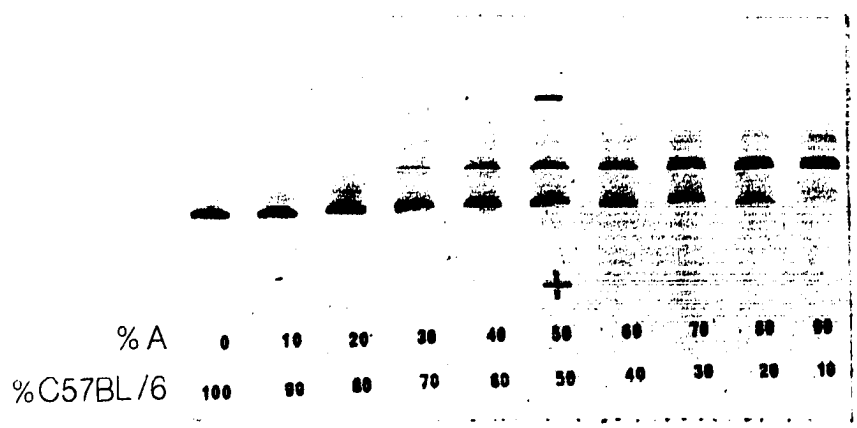


Figure 17. Polyacrylamide gel isoelectric focusing patterns of known mixtures of C57BL/6 and A hemoglobins

hemoglobins in F_1 mice are expressed in a codominant fashion. Each gel shown in Figure 16 had the same total amount of hemoglobin (350 μg) applied to the gel. Therefore, the concentration of both the A and SJL components of the (A x SJL)F_1 hybrid are present at one-half the concentration of the individual A and SJL standards. At this concentration the minor component of the A hemoglobin from the (A x SJL)F_1 hybrid cannot be seen.

Figure 17 shows the isoelectric focusing gels of various mixtures of C57BL/6 and A hemoglobins. It is seen that this technique resolves the diffuse (double) hemoglobin of the A mice into a major band and a minor band, and that under proper conditions both bands migrate to a different point than the single band from the C57BL/6 mice. This is a marked advantage of this isoelectric focusing technique compared to other electrophoretic techniques which have been used in the past. It is this separation of the diffuse (double) and single hemoglobins that has allowed the quantitation of mixtures of the two hemoglobin types.

The analysis of artificial mixtures of C57BL/6 hemoglobin with (A x SJL)F_1, (CBA x CBA/H-T6)F_1, DBA/1, A and C57BL/10Sn hemoglobin with A is shown in Table 11. Each of the data forms a standard curve from which the unknown composition of an individual allophenic mouse may be calculated. Representative standard curves for C57BL/6 hemoglobin with A and C57BL/10Sn hemoglobin with A are shown in Figures 18 and 19, respectively. These standard curves were formed after gels were scanned at a wavelength of 576 nm and the area under the C57BL/6 or C57BL/10Sn peak

Table 11. Analysis of artificial mixtures of hemoglobins

A. C57BL/6 and A x SJL

Theoretical Percentages			Number	Experimental Amount A
% C57BL/6	%AxSJL	% A of Total	Determinations	(Mean ± S.D.)
100	0	0	--	--
90	10	5	18	2 ± 6
75	25	12.5	12	14 ± 14
50	50	25	16	27 ± 10
25	75	37.5	8	34 ± 10
10	90	45	4	43 ± 4
0	100	50	15	51 ± 7

B. C57BL/6 and CBA

Theoretical Percentages		Number	Experimental Amount
% C57BL/6	% CBA	Determinations	C57BL/6 (Mean ± S.D.)
100	0	16	103 ± 7
90	10	14	87 ± 5
75	25	12	69 ± 8
60	40	6	58 ± 3
50	50	17	51 ± 8
25	75	16	28 ± 8
10	90	6	14 ± 3
0	100	--	--

Table 11. (continued)

C. C57BL/6 and DBA/1

Theoretical Percentages		Number	Experimental Amount
% C57BL/6	%DBA/1	Determinations	C57BL/6 (Mean ± S.D.)
100	0	6	102 ± 3
90	10	4	81 ± 3
75	25	5	69 ± 3
60	40	2	57 ± 4
50	50	6	52 ± 6
25	75	6	29 ± 4
10	90	2	17 ± 3
0	100	--	--

D. C57BL/6 and A

Theoretical Percentages		Number	Experimental Amount
% C57BL/6	% A	Determinations	C57BL/6 (Mean ± S.D.)
100	0	2	100 ± 13
90	10	2	75 ± 21
80	20	2	86 ± 10
70	30	2	69 ± 6
60	40	2	66 ± 8
50	50	2	54 ± 9
40	60	2	48 ± 13

Table 11. (continued)

D. C57BL/6 and A

Theoretical Percentages			
% C57BL/6	% A	Number Determinations	Experimental Amount C57BL/6 (Mean ± S.D.)
30	70	2	25 ± 4
20	80	2	16 ± 2
10	90	2	6 ± 2
0	100	--	--

E. C57BL/10Sn and A

Theoretical Percentages			
% C57BL/10Sn	% A	Number Determinations	Experimental Amount C57BL/10Sn (Mean ± S.D.)
100	0	2	92 ± 8
90	10	2	91 ± 2
80	20	2	82 ± 5
70	30	2	69 ± 1
60	40	2	68 ± 5
50	50	2	48 ± 1
40	60	2	39 ± 7
30	70	2	29 ± 1
20	80	2	22 ± 1
10	90	2	7.5 ± 1
0	100	--	--

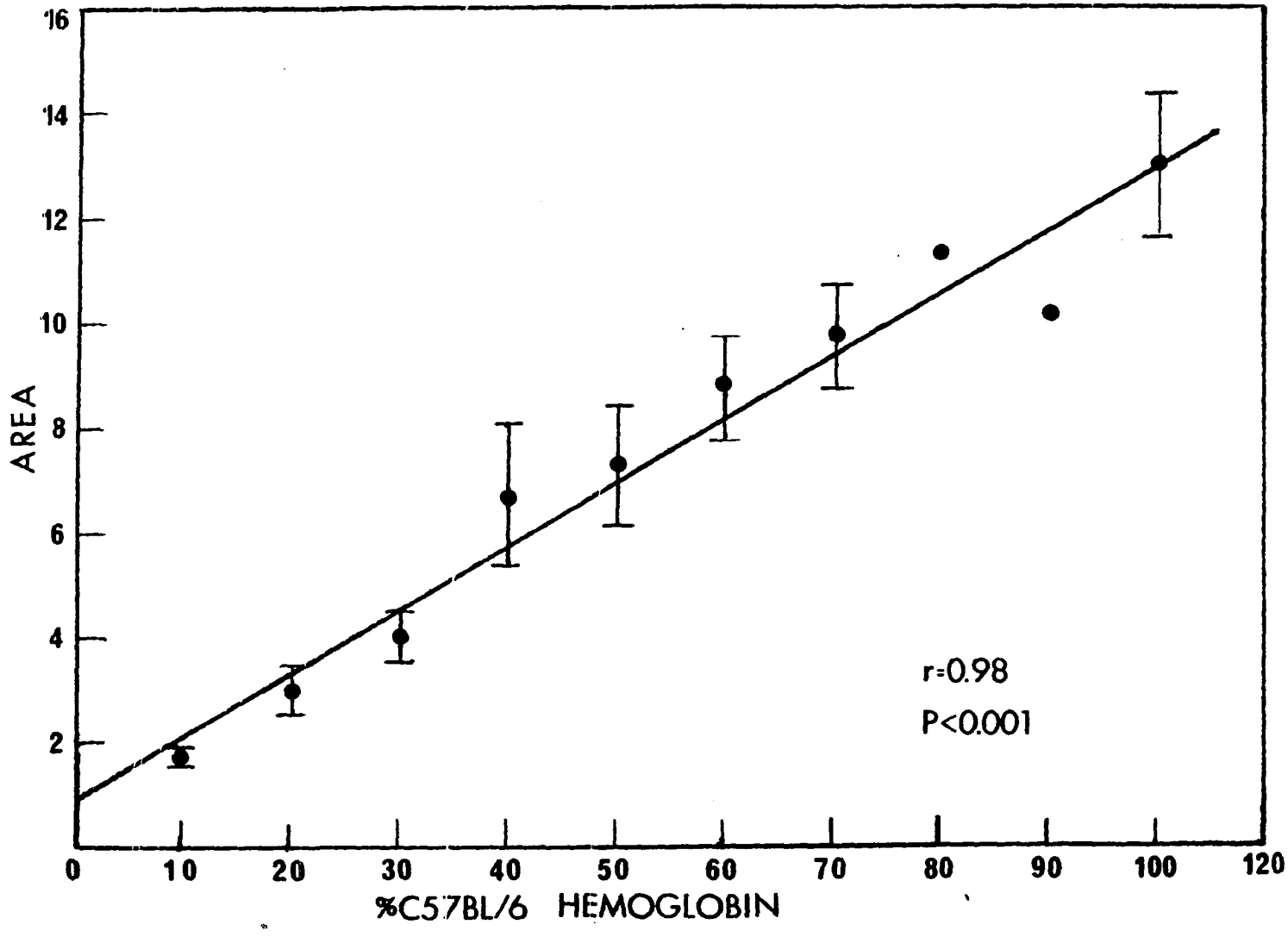


Figure 18. The area under the C57BL/6 peak as a function of the percentage of C57BL/6 hemoglobin

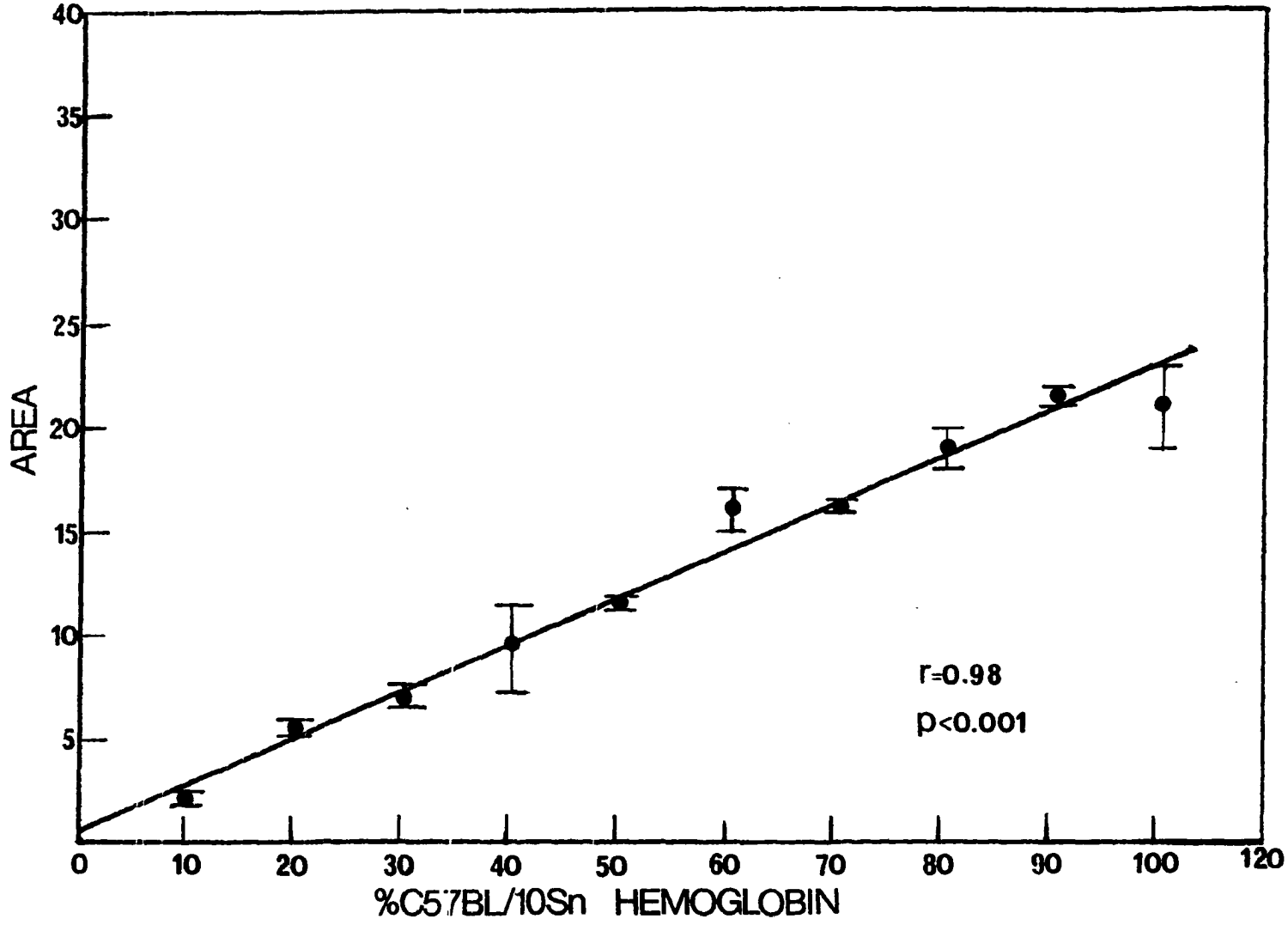


Figure 19. The area under the C57BL/10Sn peak as a function of the percentage of C57BL/10Sn hemoglobin

was plotted as a function of the percentage of single type hemoglobin applied on the gel. The compositions of the unknown allophenic mice of these combinations were determined by comparing the area under the single type hemoglobin peak with the standard curve.

It can be seen in Table 11 that for the C57BL/6 with CBA and C57BL/6 with DBA/1 mixtures, there is a definite tendency for the amount of C57BL/6 hemoglobin to be overestimated when it is less than or equal to 10% of the total mixture. The relatively large error in the C57BL/6 with (A x SJL)F_1 mixtures is due to the fact that the C57BL/6 and SJL hemoglobins comigrate in the gels. Thus, these gels must be quantitated for the percent A hemoglobin, and since this value can never be more than 50% of the total, there is an increase in the error, especially at the points with a low percent of A hemoglobin compared to the total.

2. Isoelectric points of murine hemoglobins

Figure 20 illustrates the pH gradient formed during a four hour electrophoresis using a 7% polyacrylamide gel containing a total of 2% (3-10)(6-8) ampholytes and 5% glycerol. The standard curve from which unknown isoelectric points could be estimated was established by drawing a least squares line through the measured pH values as a function of gel length. The good linearity observed in the pH range of 5.5-8.0 is attributed to the enrichment with ampholytes (6-8) in that part of the gel. Table 12 summarizes the isoelectric point values of hemoglobin samples from six inbred strains of mice and (A x SJL)F_1 hybrids before and after

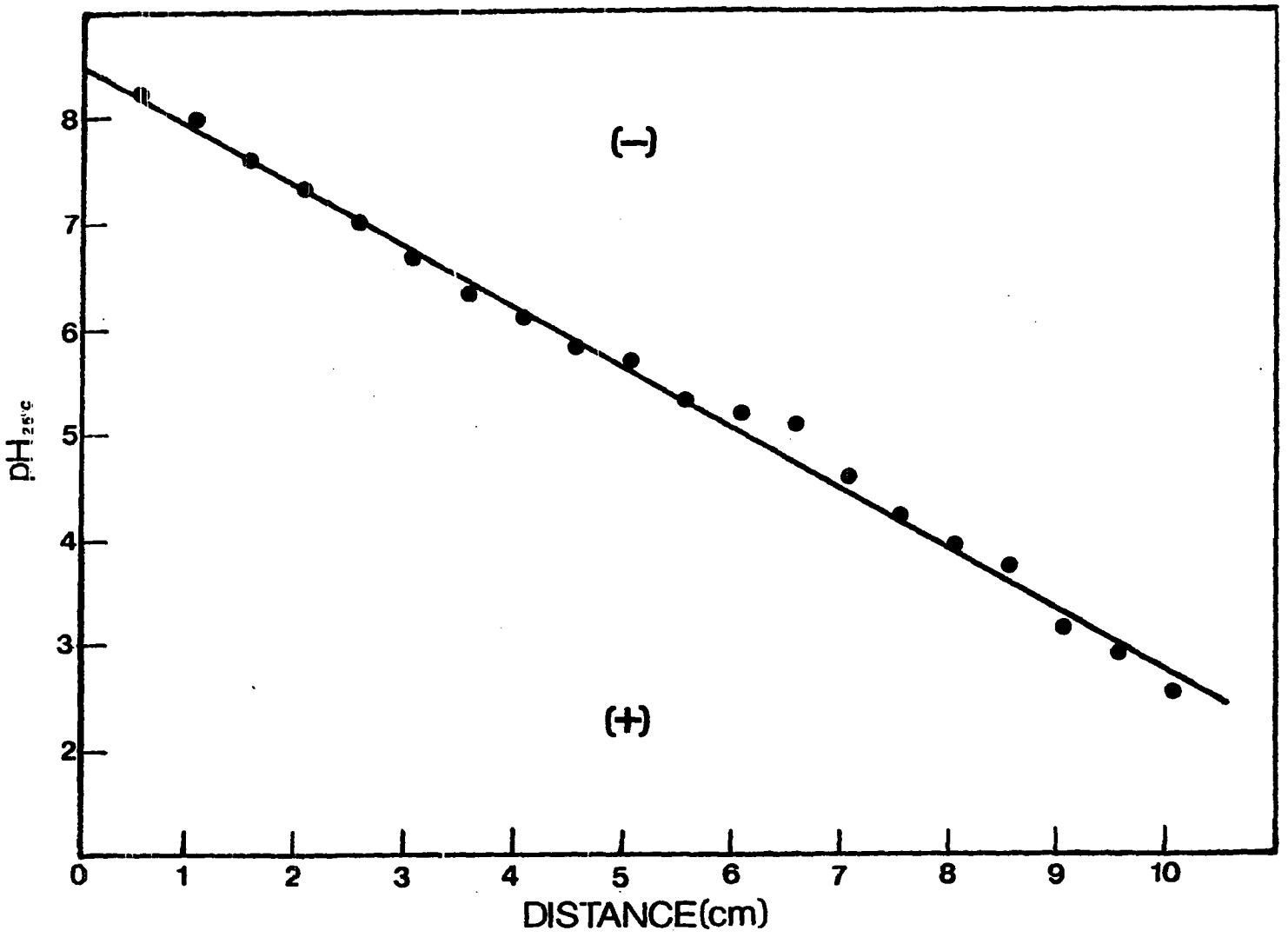


Figure 20. pH gradient obtained in 10 cm polyacrylamide gel containing 2% ampholytes (3-10)(6-8) and 5% glycerol. [pH measurements were made at room temperature.]

Table 12. Isoelectric points of hemoglobins from inbred and F_1 hybrid mice

Mouse Type	Hemoglobin Type[b]	Isoelectric Points (pI)[a]					
		Before Chemical Modification			After Chemical Modification		
		Single Type	Diffuse(double) Type		Single Type	Diffuse(double) Type	
		Major Band	Major Band	Minor Band	Major Band	Major Band	Minor Band
Inbred Strains							
C57BL/6	Single	7.33 ± .02	--	--	7.52 ± .02	--	--
C57BL/10Sn	Single	7.33 ± .02	--	--	7.52 ± .02	--	--
SJL	Single	7.30	--	--	7.55	--	--
A	Diffuse (double)	--	7.48 ± .08	7.58 ± .02		7.75	7.86
CBA	Diffuse (double)	--	7.45 ± .05	7.60		7.75	7.86
DBA/1	Diffuse (double)	--	7.48 ± .05	7.63 ± .02		7.75	7.86
F_1 Hybrid							
(A x SJL)F_1	Diffuse (double)-Single	7.4 ± .10	7.5 ± .02	7.63 ± .02	7.58 ± .02	7.73 ± .02	7.84 ± .03

[a]Isoelectric point estimations of hemoglobins are based on duplicate determinations and values are reported as pI ± standard deviation.

[b]This refers to the genetic polymorphism in Hbb (Hutton et al., 1962a,b; Gilman, 1972). Electrophoresis shows Hbb either as a "single" or as a "diffuse" species. In this study, isoelectric focusing further defines Hbb as two bands, which is referred to as double hemoglobin.

chemical modification with cystamine dihydrochloride. In Table 12, it can be seen that the single Hbb(C57BL/6, C57BL/10Sn, SJL) and diffuse Hbb(A, CBA, DBA/1) have different isoelectric points (pI) before and after chemical modification. However, after treatment with the cystamine dehydrochloride, the hemoglobin resolution of single and double mixtures, as shown in (A x SJL)F_1 mice, are optimized. This is achieved by the addition of an S-ethylamine group to the cysteine at position 13 of the double Hbb type, while the glycine at the same position in the single Hbb type remains unchanged (see Figure 16) (Wegmann and Gilman, 1970). In addition, it can be seen that there are no significant differences in the pI values of hemoglobins of the single Hbb type among strains of mice. This observation is also true for the diffuse Hbb type mouse strains. This suggests that any possible substitutions existing between mouse strains of the same Hbb type are conserved, and therefore, hemoglobins of a type are electrophoretically identical.

F. Analysis of Changes in Red Blood Cell and White Blood Cell Populations in Allophenic Mice

The results of hemoglobin and peripheral white blood cell determinations on 28 C57BL/6 ⟷ (A x SJL)F_1, 15 (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, 4 C57BL/6 ⟷ DBA/1, 15 C57BL/6 ⟷ A, and 9 C57BL/10Sn ⟷ A allophenic mice are summarized in Tables 13, 14, 15, 16, and 17, and shown in Figures 21, 22, 23, 24, and 25, respectively. It is seen that Figures 21, 22, and 23 consist of two sets of data. In the first set, hemoglobin and peripheral white blood cell composition were tested twice, at an interval

Table 13. Analysis of C57BL/6 ⟷ (A x SJL)F_1 allophenic mice

Mouse Number	Percentage of C57BL/6 PWBC or Hb[a]					
	First Determination		Second Determination		Third Determination	
	PWBC	Hb	PWBC	Hb	PWBC	Hb
83	48 ± 0	17	10 ± 0	21 ± 1	--	--
84	63 ± 0	40 ± 9	16 ± 2	0 ± 0	--	--
85	56 ± 0	100 ± 0	29 ± 4	37	--	--
86	10 ± 0	31 ± 2	6 ± 1	7	--	--
87	-6 ± 0	14 ± 14	46 ± 2	14 ± 14	--	--
98	44 ± 2	58 ± 7	4 ± 0	58 ± 8	--	--
108	40 ± 8	52	7 ± 1	34	--	--
109	8 ± 3	10	10 ± 2	3	--	--
110	84 ± 5	100 ± 0	79 ± 2	100 ± 0	--	--
111	13 ± 3	15 ± 9	11 ± 2	8	--	--
112	46 ± 5	54 ± 13	32 ± 4	34	--	--
113	-11 ± 5	0 ± 0	-5 ± 4	34 ± 13	--	--
114	27 ± 6	1 ± 1	16 ± 3	58 ± 3	--	--
115	87 ± 0	90 ± 10	94 ± 4	87 ± 13	--	--
116	0 ± 0	3 ± 3	12 ± 2	3 ± 3	--	--
117	-4 ± 5	34	13 ± 3	21 ± 9	--	--
118	91 ± 4	92	93 ± 1	90	--	--
132	--	20 ± 18	--	21 ± 4	--	9 ± 1
133	--	0 ± 0	--	8 ± 1	--	10 ± 10
134	--	27 ± 6	--	18 ± 18	--	11 ± 11
135	--	0 ± 0	--	1 ± 1	--	0 ± 0
137	--	33 ± 4	--	0 ± 0	--	43 ± 20
138	--	39 ± 2	--	39 ± 10	--	23 ± 7
139	--	37 ± 0	--	35 ± 8	--	35 ± 1
140	--	27 ± 11	--	14 ± 13	--	24 ± 1
141	--	31 ± 1	--	32 ± 4	--	30 ± 11
142	--	50 ± 0	--	40 ± 7	--	50 ± 2
143	--	100 ± 0	--	47 ± 1	--	37 ± 5

[a]The percentage of C57BL/6 peripheral white blood cells (PWBC) or of hemoglobin (Hb) was determined as described in the Materials and Methods section.

Percentage of C57BL/6 PWBC or Hb[a]							
Fourth Determination		Fifth Determination		Sixth Determination		Seventh Determination	
PWBC	Hb	PWBC	Hb	PWBC	Hb	PWBC	Hb
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
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--	--	--	--	--	--	--	--
1 ± 0	18 ± 12	3 ± 3	24 ± 4	--	3 ± 3	--	--
3 ± 2	16 ± 4	--	1 ± 1	1 ± 0	0 ± 0	1 ± 0	3 ± 2
1 ± 1	21 ± 4	--	14 ± 10	3 ± 0	0 ± 0	3 ± 2	10 ± 5
7 ± 1	3 ± 3	--	13 ± 2	0 ± 0	7 ± 0	2 ± 2	13 ± 8
3	23 ± 3	--	20 ± 0	2 ± 1	0 ± 0	1 ±1	11 ± 7
3 ± 1	45 ± 5	--	9 ± 5	2 ± 0	27 ± 2	8 ± 1	28 ± 4
6 ± 6	29 ± 6	--	26 ± 3	1 ± 1	25 ± 0	6 ± 1	10 ± 5
11 ± 1	34 ± 7	--	41 ± 7	0 ± 0	21	2 ± 0	30 ± 10
11 ± 1	22 ± 3	--	29 ± 1	0 ± 0	30 ± 0	1	20 ± 3
12 ± 7	43 ± 9	--	72 ± 0	7 ± 2	50 ± 0	23 ± 3	46 ± 0
24 ± 5	50 ± 2	--	42 ± 3	19 ± 3	45 ± 3	24 ± 5	41 ± 0

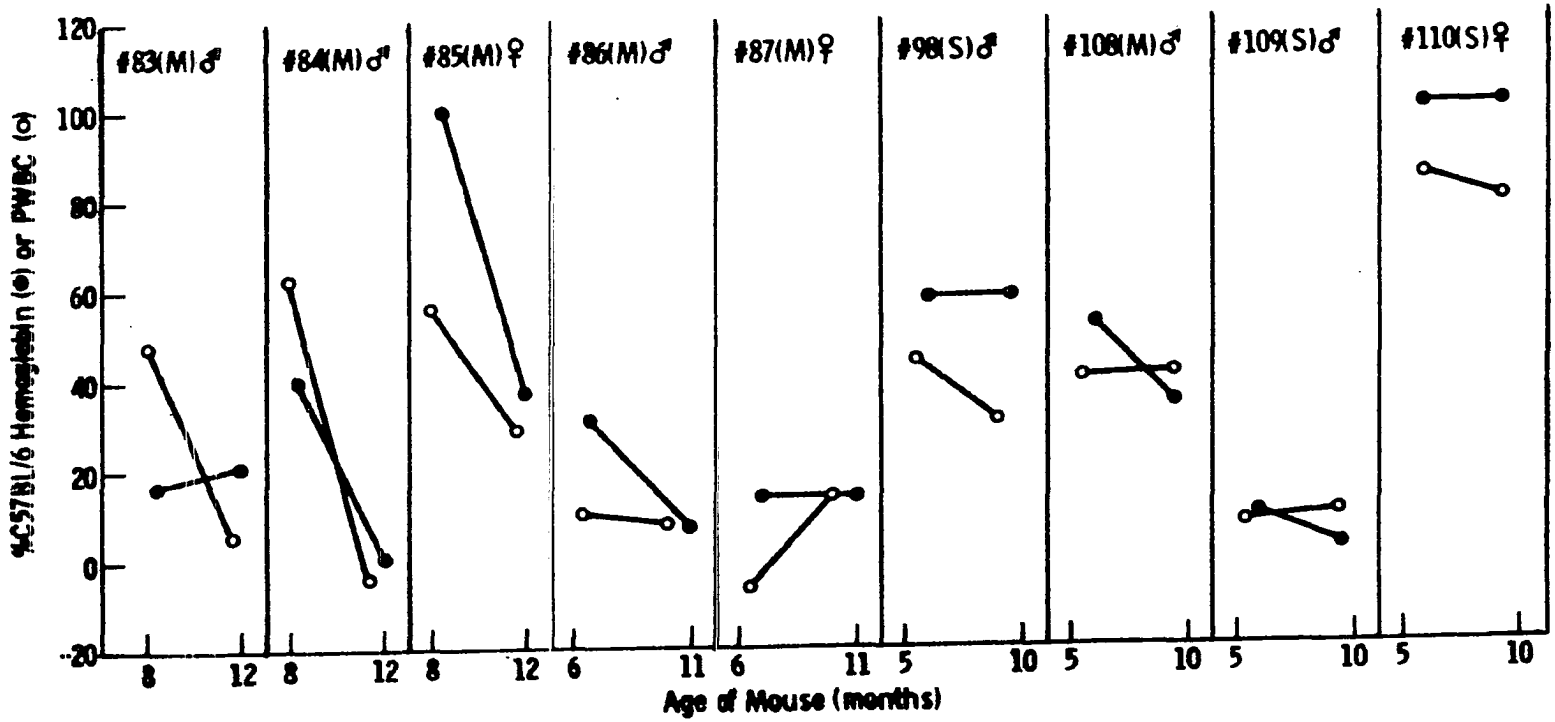


Figure 21. Hemoglobin and peripheral white blood cells (PWBC) composition of C57BL/6 ⟷ (AxSJL)F_1 mice as a function of age of mice. [S and M refers to single- and multi-colored mice, respectively.]

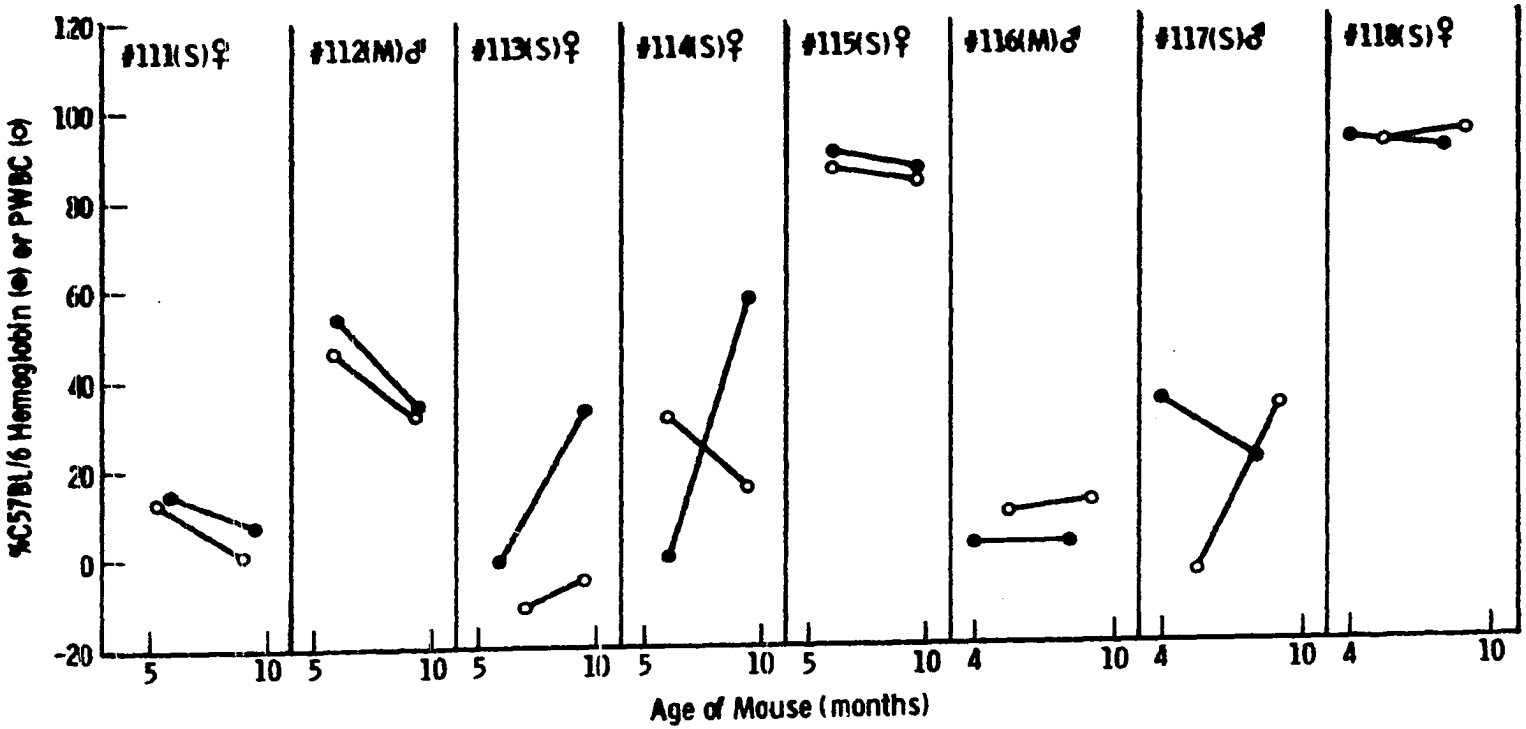


Figure 21. (continued)

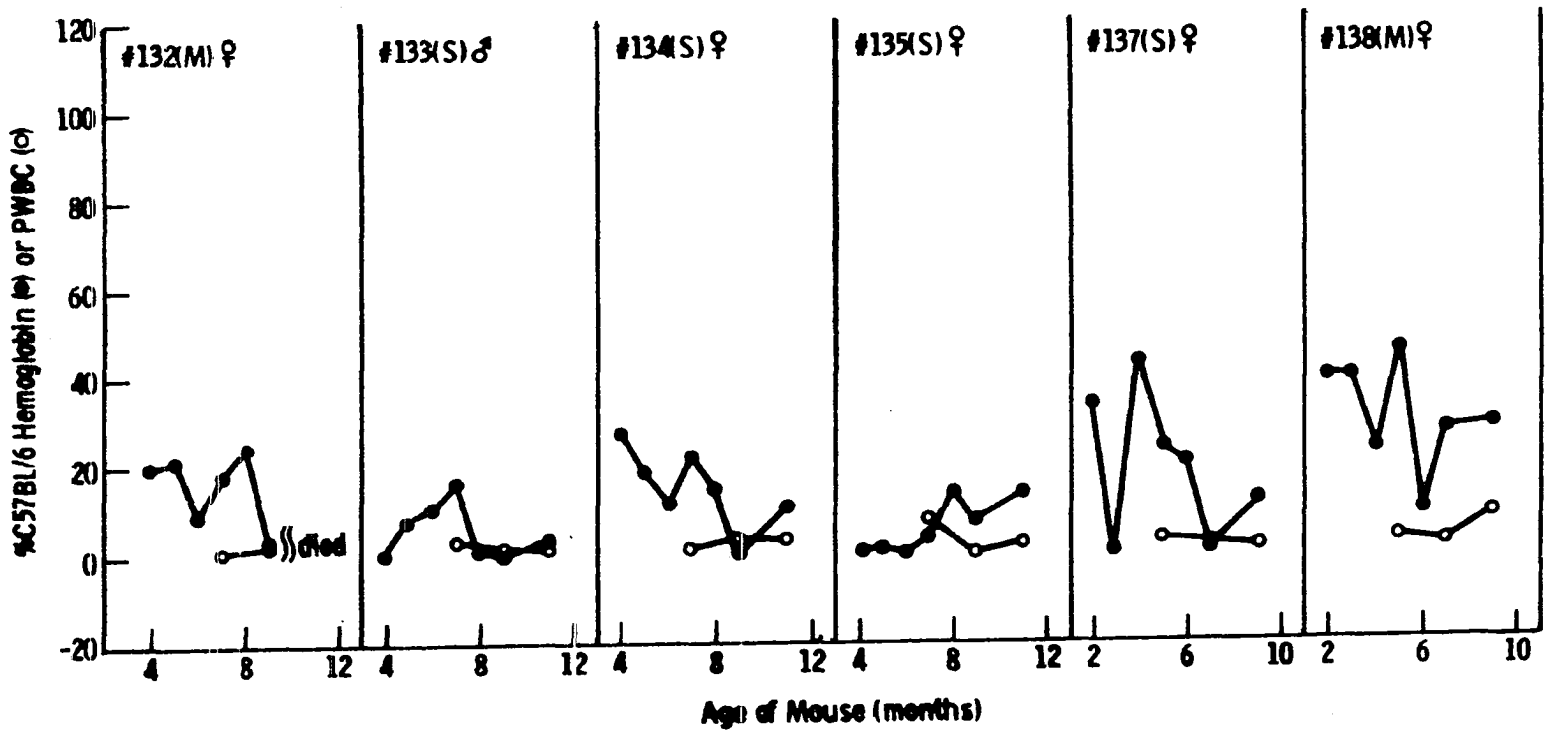


Figure 21. (continued)

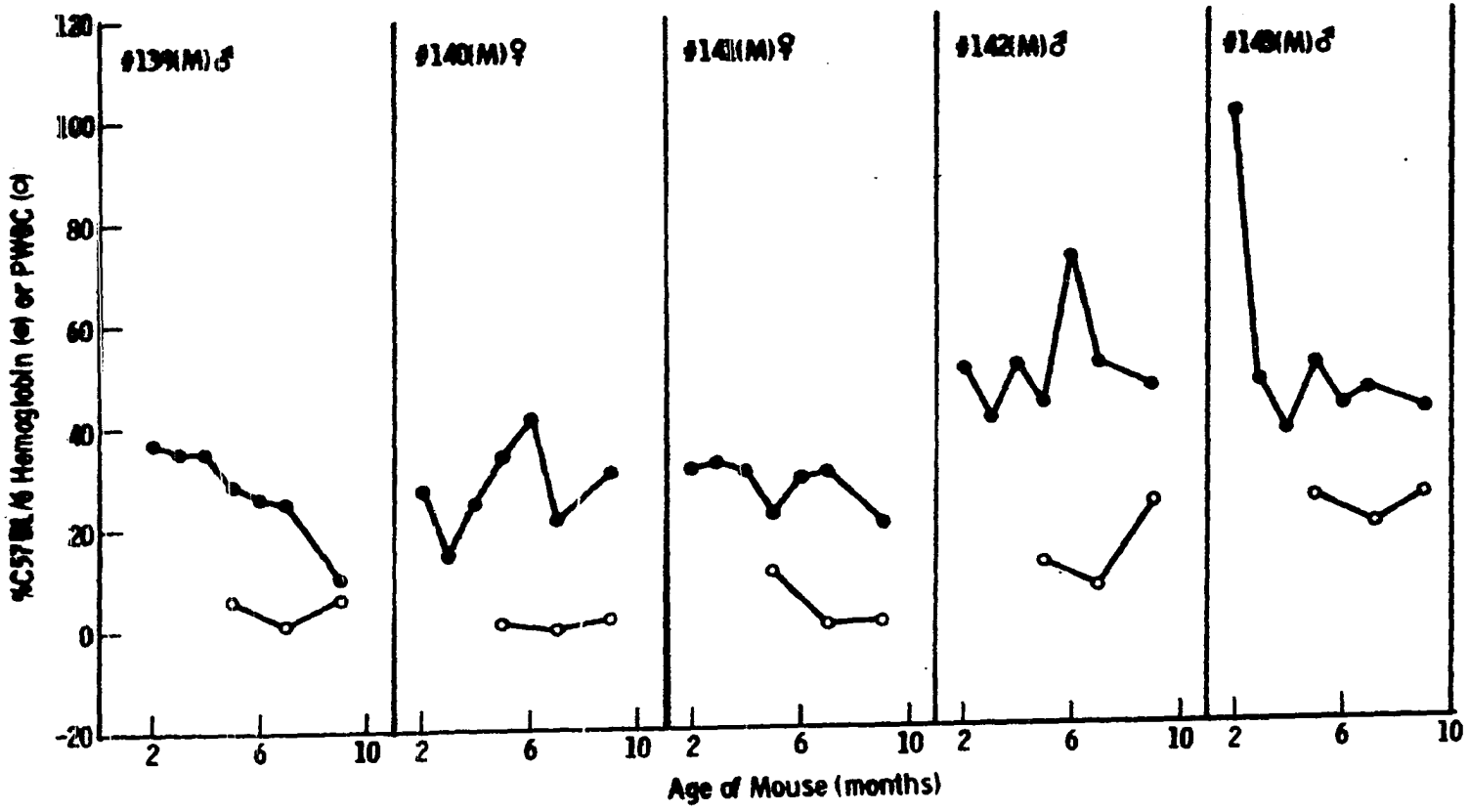


Figure 21. (continued)

Table 14. Analysis of (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 allophenic mice

Mouse Number	Percentage of C57BL/6 PWBC or Hb[a]					
	First Determination		Second Determination		Third Determination	
	PWBC	Hb	PWBC	Hb	PWBC	Hb
74	71 ± 2	101	99 ± 1	101	--	--
76	84 ± 4	35	85 ± 0	106	--	--
80	74 ± 3	79	103 ± 1	101	--	--
81	86 ± 5	83	105 ± 1	100	--	--
82	47 ± 5	103	61 ± 0	103	--	--
119	77 ± 2	108	66 ± 3	53	--	--
120	27 ± 3	45	41 ± 2	33	--	--
121	79 ± 2	113	62 ± 4	110	--	--
125	--	105	--	108	--	103 ± 5
126	--	50	--	44	--	62
127	--	56 ± 2	--	74 ±13	--	59 ± 2
128	--	83 ± 3	--	83	--	83 ± 8
129	--	59 ± 6	--	51 ± 4	--	66 ± 2
130	--	20 ± 1	--	8 ± 1	--	6 ± 2
131	--	23	--	15 ± 2	--	13

[a]The percentage of C57BL/6 peripheral white blood cells (PWBC) or of hemoglobin (Hb) was determined as described in the Materials and Methods section.

Percentage of C57BL/6 or Hb[a]							
Fourth Determination		Fifth Determination		Sixth Determination		Seventh Deterimantion	
PWBC	Hb	PWBC	Hb	PWBC	Hb	PWBC	Hb
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
--	--	--	--	--	--	--	--
93 ± 1	93 ± 1	--	98 ± 1	74 ± 2	87 ± 4	83 ± 1	90
52 ± 2	58 ± 2	--	74 ± 3	48 ± 0	69 ± 2	72 ± 0	82 ± 5
88 ± 1	61 ± 2	--	56	60 ± 10	71 ± 3	69 ± 1	92 ± 1
93 ± 5	94 ± 6	--	79 ± 4	31 ± 1	77 ± 2	52 ± 0	85
8 ± 2	53 ± 3	--	81 ± 6	20 ± 1	65 ± 3	19 ± 6	78
0	9 ± 1	--	6	0	0 ± 0	0 ± 0	0 ± 0
0	12	--	10 ± 2	1 ± 1	2	1 ± 0	4

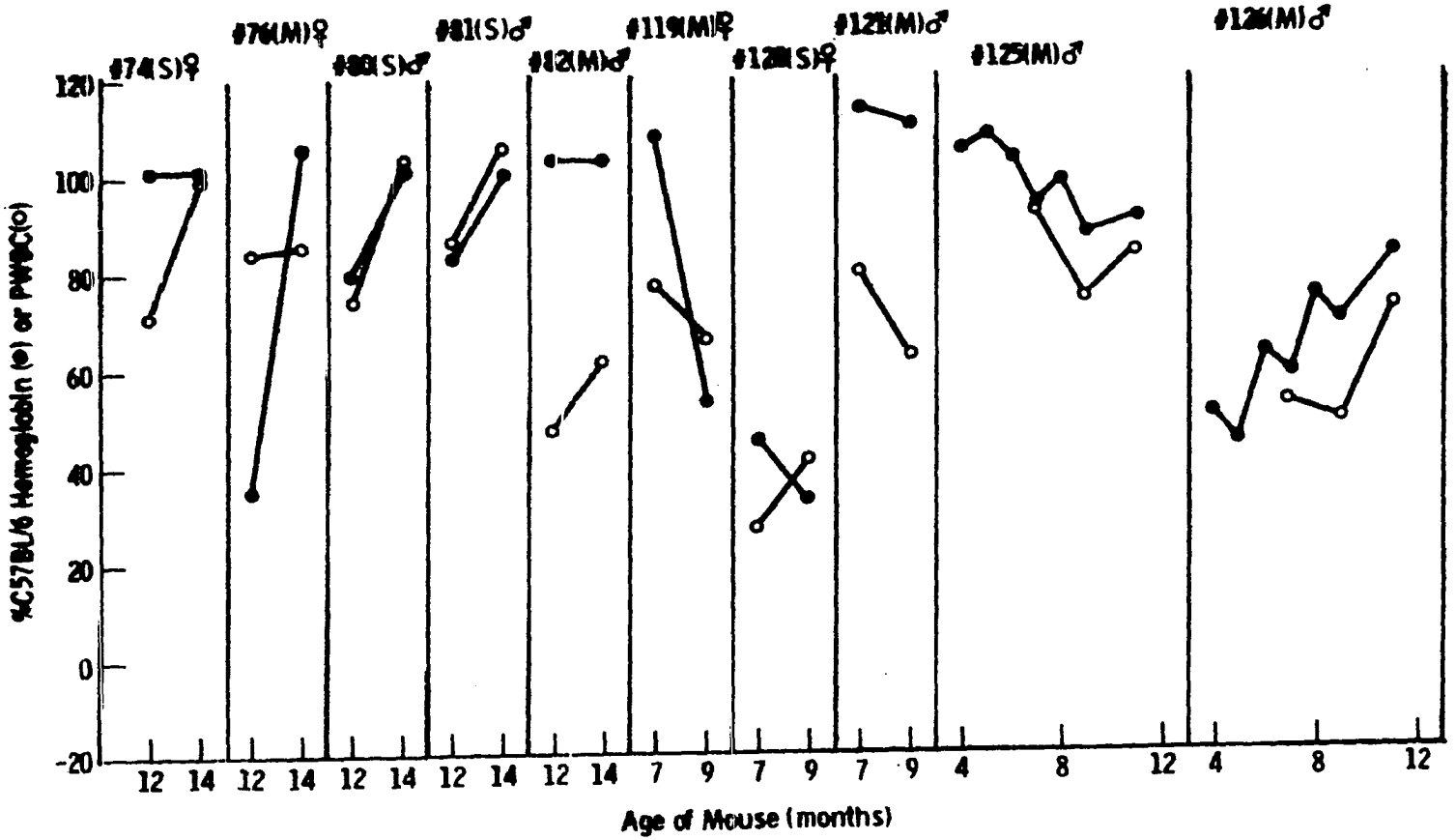


Figure 22. Hemoglobin and peripheral white blood cells (PWBC) composition of (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 mice as a function of age of mice. [S and M refer to single- and multi-colored mice, respectively.]

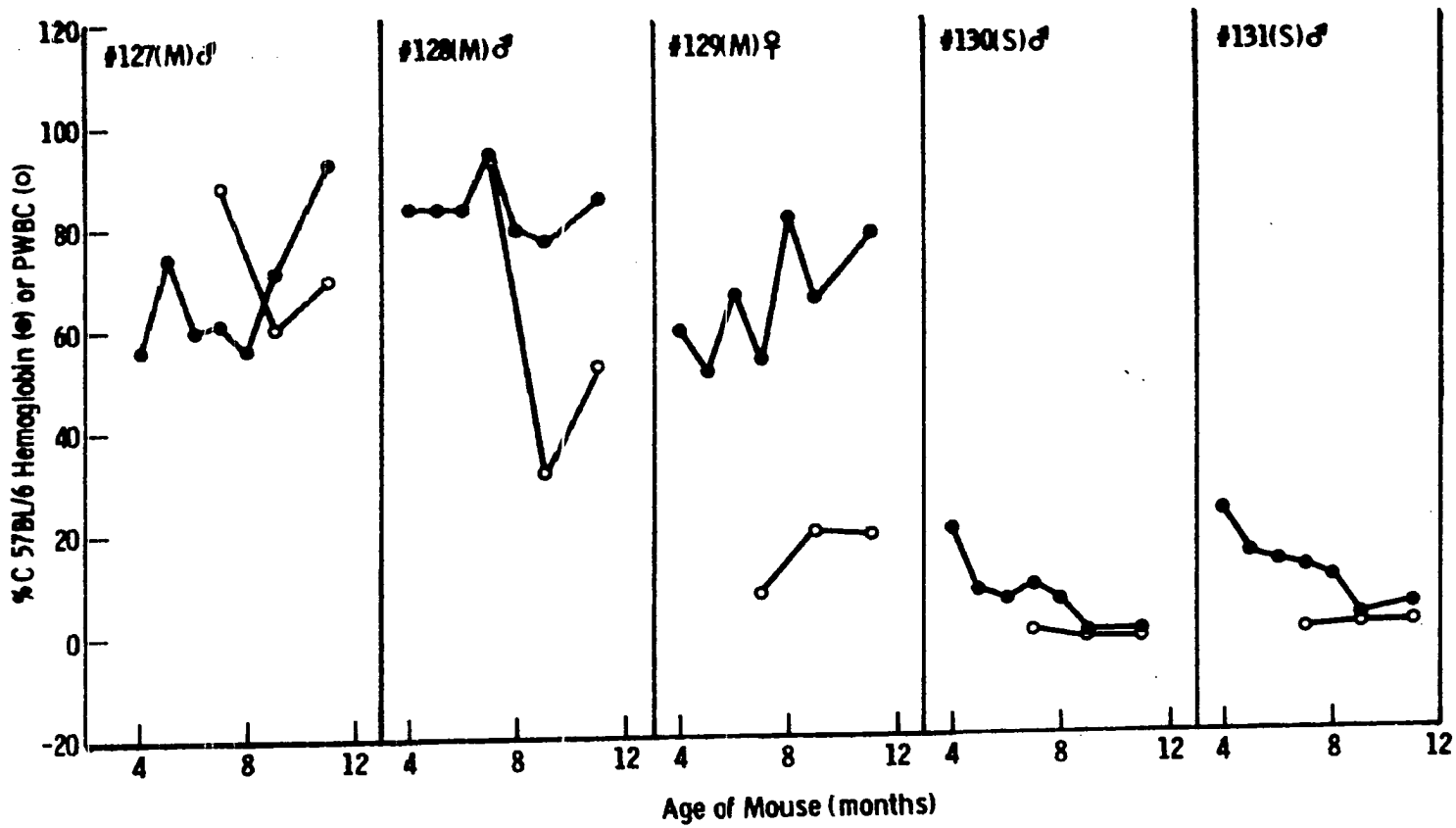


Figure 22. (continued)

Table 15. Analysis of C57BL/6 ⟷ DBA/1 allophenic mice

Mouse Number	Percentage of C57BL/6 PWBC or Hb[a]					
	First Determination		Second Determination		Third Determination	
	PWBC	Hb	PWBC	Hb	PWBC	Hb
101	15 ± 0	6	13 ± 0	10	--	--
122	--	25 ± 5	--	4	--	4 ± 2
123	--	91	--	108 ± 2	--	96 ± 3
124	--	66 ± 6	--	72	--	50

[a]The percentage of C57BL/6 peripheral white blood cells (PWBC) or hemoglobin (Hb) was determined as described in the Materials and Methods section.

Percentage of C57BL/6 PWBC or Hb[a]							
Fourth Determination		Fifth Determination		Sixth Determination		Seventh Determination	
PWBC	Hb	PWBC	Hb	PWBC	Hb	PWBC	Hb
--	--	--	--	--	--	--	--
0 ± 0	4 ± 2	--	--	--	--	--	--
70 ± 3	101 ± 1	--	110 ± 7	43 ± 2	107	79 ± 3	100
43 ± 0	54	--	57	34 ± 3	52 ± 1	69 ± 2	55 ± 3

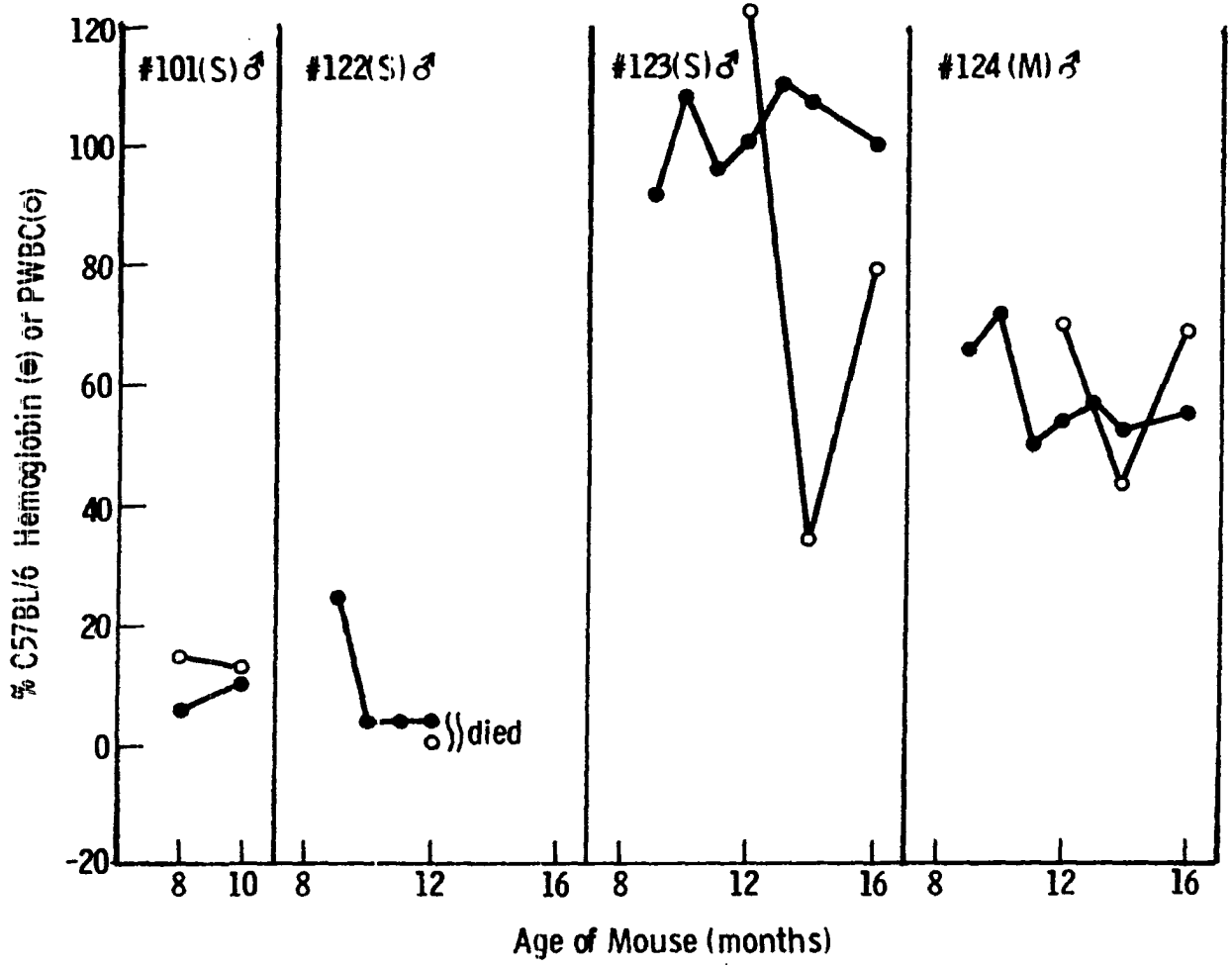


Figure 23. Hemoglobin and peripheral white blood cells (PWBC) composition of C57BL/6 ⟷ DBA/1 mice as a function of age of mice. [S and M refer to single- and multi-colored mice, respectively.]

Table 16. Analysis of C57BL/6 ⟷ A allophenic mice

Mouse Number	Percentage of C57BL/6 PWBC or Hb[a]					
	First Determination		Second Determination		Third Determination	
	PWBC	Hb	PWBC	Hb	PWBC	Hb
170	4 ± 1	5 ± 2	3 ± 1	6 ± 1	4 ± 1	0 ± 0
159	32 ± 7	49 ± 6	48 ± 2	33 ± 0	14 ± 3	82
162	59 ± 1	69 ± 3	66 ± 6	70 ± 0	27 ± 6	66 ± 4
171	28 ± 1	41 ± 5	32 ± 1	15 ± 3	8 ± 1	12
191	42 ± 4	51 ± 9	28 ± 0	51 ± 21	11 ± 3	7 ± 2
169	60 ± 8	76 ± 3	61 ± 4	65 ± 10	62 ± 2	58
172	56 ± 6	73	46 ± 4	54	36 ± 0	43 ± 2
173	71 ± 1	76 ± 4	58 ± 4	79 ± 1	25 ± 0	57
168	102 ± 0	100	113 ± 7	99 ± 5	102 ± 4	89 ± 3
158	99 ± 9	95 ± 2	44 ± 4	72 ± 3	107 ± 1	98 ± 3
157	92 ± 7	96 ± 18	91 ± 2	97 ± 1	97 ± 4	89
160	90 ± 1	96 ± 2	87 ± 1	83 ± 8	57 ± 1	88
167	80 ± 6	93 ± 11	106 ± 1	92 ± 7	77 ± 1	102 ± 25
187	96 ± 1	80 ± 5	58 ± 1	95 ± 12	56 ± 5	103 ± 0
188	119 ± 5	67	79 ± 1	93 ± 25	84 ± 6	93 ± 0

[a]The percentage of C57BL/6 peripheral white blood cells (PWBC) or of hemoglobin (Hb) was determined as described in the Materials and Methods section.

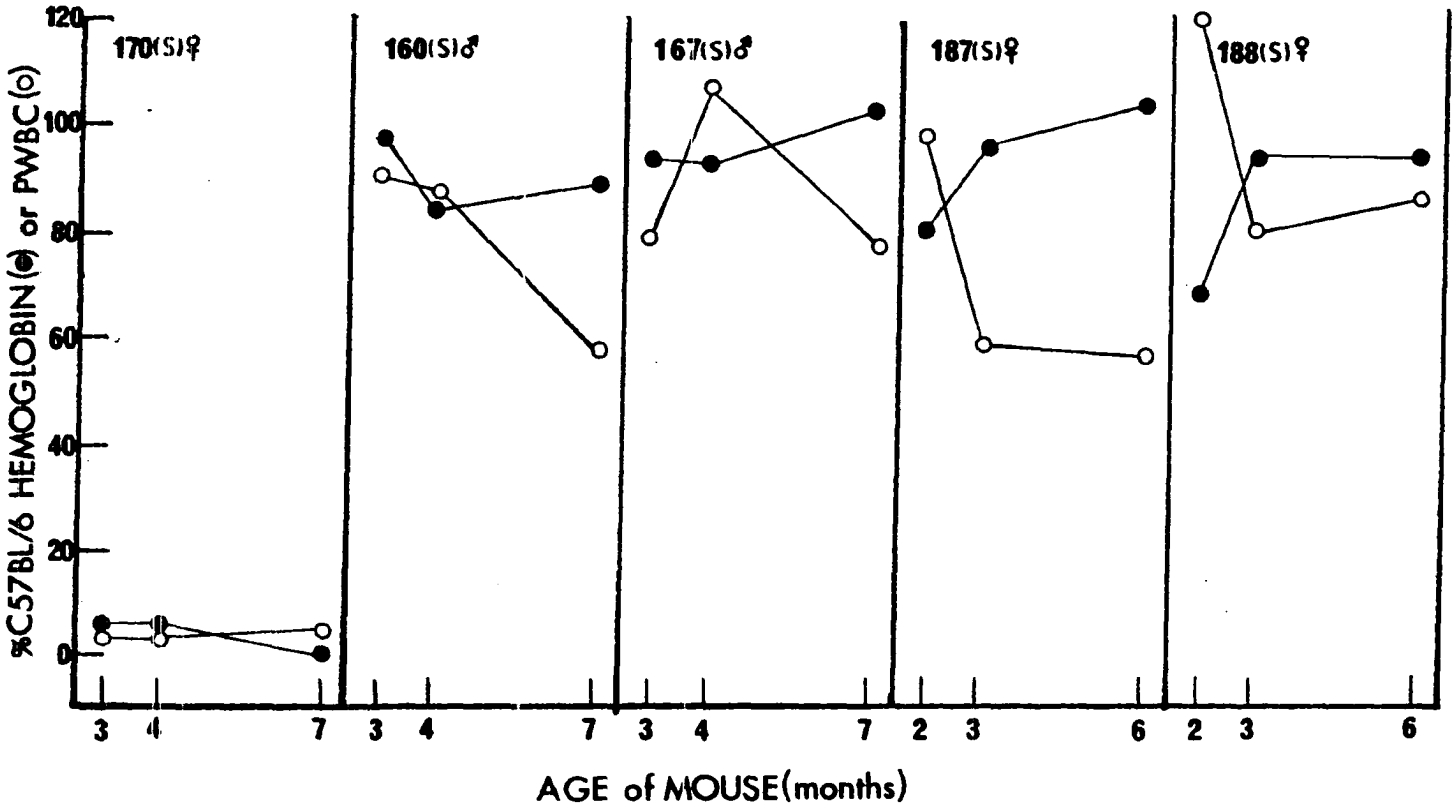


Figure 24. Hemoglobin and peripheral white blood cells (PWBC) composition of C57BL/6 ⟷ A mice as a function of age of mice. [S and M refer to single- and multicolored mice, respectively.]

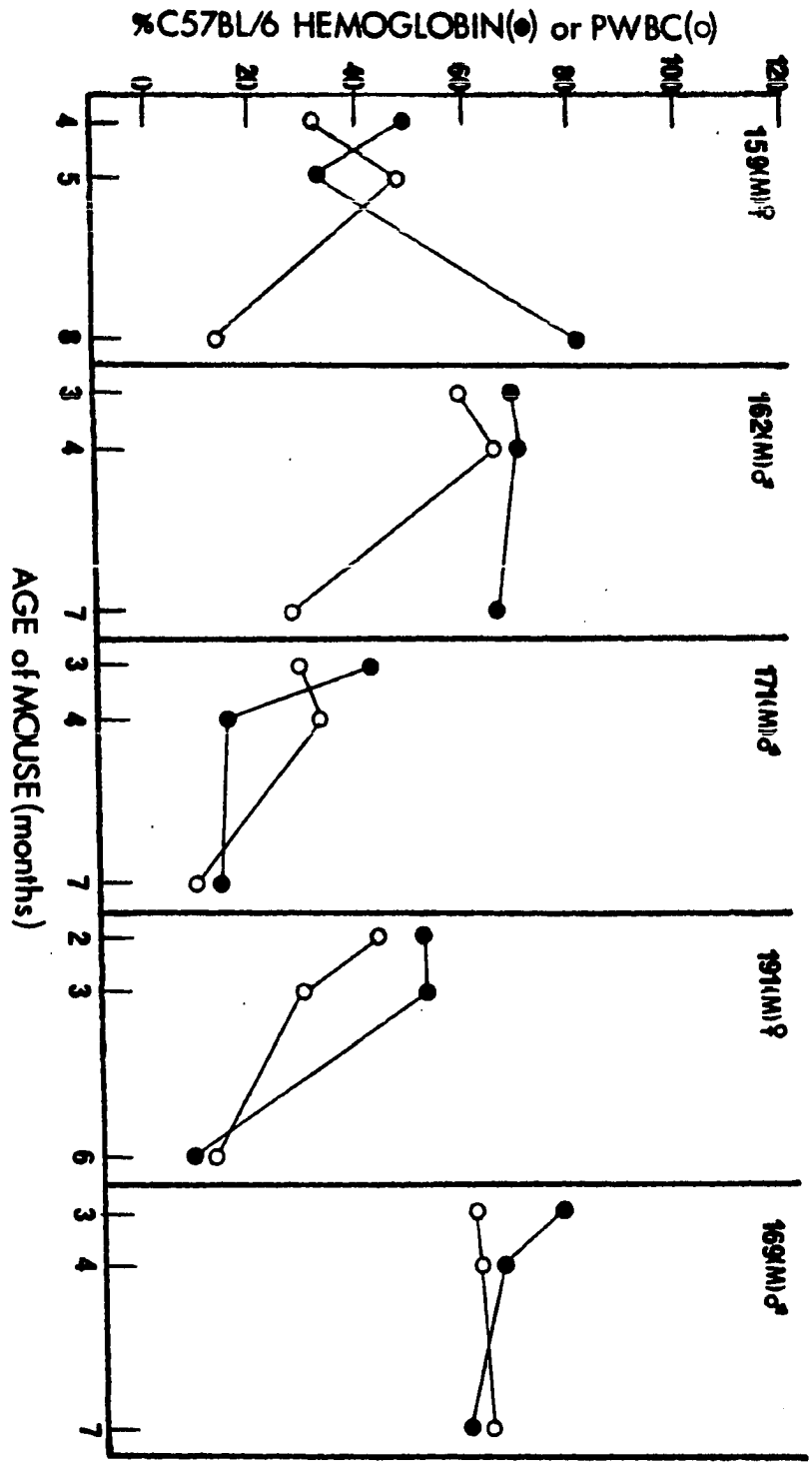


Figure 24. (continued)

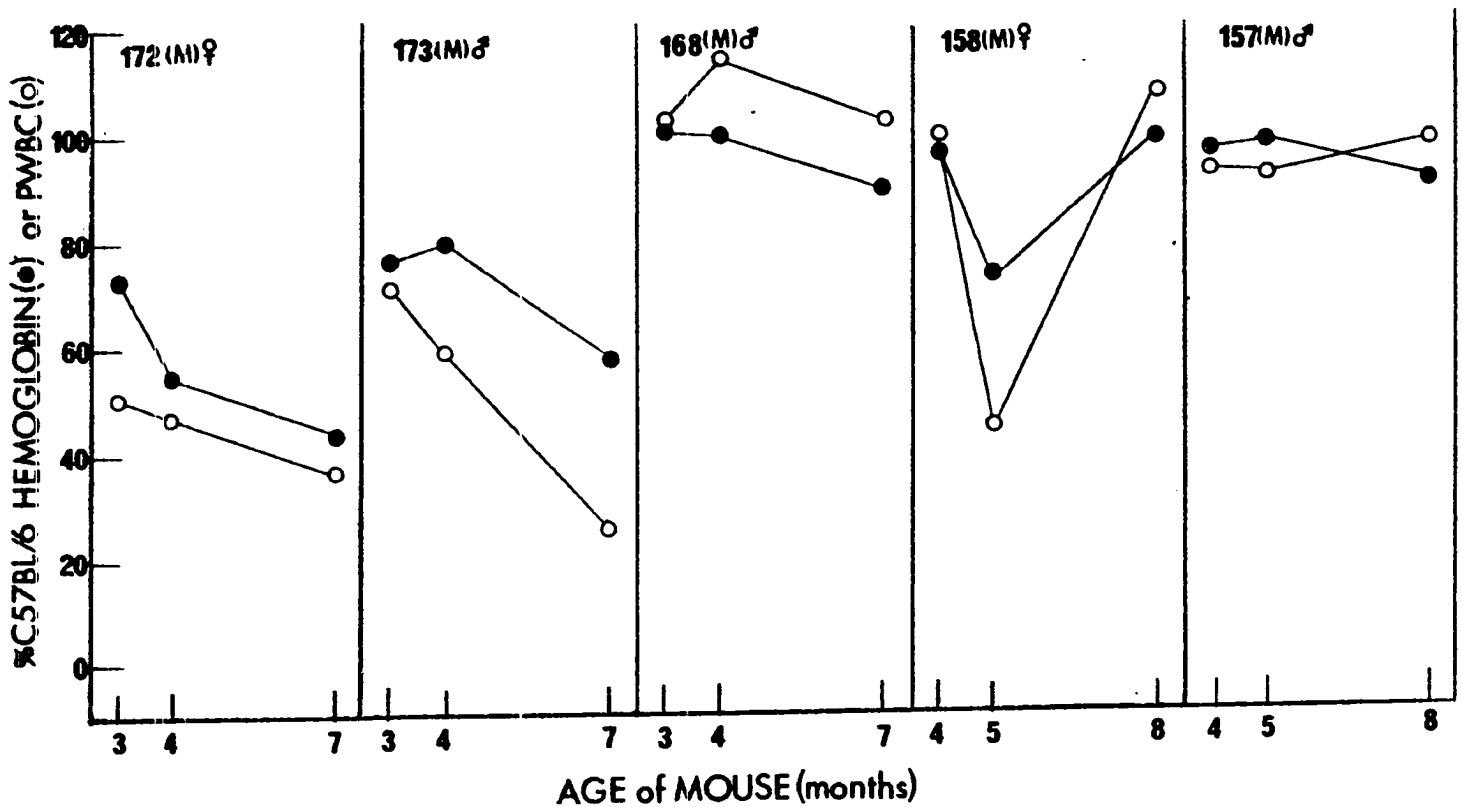


Figure 24. (continued)

Table 17. Analysis of C57BL/10Sn ⟷ A allophenic mice

Mouse Number	Percentage of C57BL/10Sn PWBC or Hb[a]					
	First Determination		Second Determination		Third Determination	
	PWBC	Hb	PWBC	Hb	PWBC	Hb
205	0 ± 0	2 ± 0	6 ± 2	2 ± 3	3 ± 1	3 ± 2
206	97 ± 0	107 ± 4	91 ± 5	77 ± 2	111 ± 0	91 ± 5
207	97 ± 2	82 ± 0	94 ± 2	82 ± 0	99 ± 2	83 ± 1
210	3 ± 3	48 ± 0	10 ± 7	54 ± 6	9 ± 2	22 ± 5
211	0 ± 0	45 ± 1	6 ± 3	35 ± 1	3 ± 1	30 ± 3
212	2 ± 1	25 ± 0	38 ± 5	27 ± 0	21 ± 6	33 ± 1
213	103 ± 5	66 ± 1	90 ± 2	67 ± 1	103 ± 2	49 ± 4
214	91 ± 5	65 ± 1	67 ± 4	58 ± 3	45 ± 0	68 ± 5
215	105 ± 3	90 ± 1	94 ± 3	105 ± 0	103 ± 6	74 ± 6

[a]The percentage of C57BL/10Sn white blood cells (PWBC) or of hemoglobin (Hb) was determined as described in the Materials and Methods section.

Percentage of C57BL/10Sn PWBC or Hb[a]					
Fourth Determination		Fifth Determination		Sixth Determination	
PWBC	Hb	PWBC	Hb	PWBC	Hb
2 ± 5	0 ± 0	15 ± 6	1 ± 1	0 ± 0	2 ± 0
104 ± 0	101 ± 2	95 ± 1	75 ± 0	100 ± 2	100 ± 0
103 ± 1	78 ± 9	94 ± 0	63 ± 3	96 ± 2	74 ± 2
2 ± 1	49 ± 1	0 ± 0	37 ± 2	1 ± 1	36 ± 1
9 ± 8	41 ± 1	0 ± 0	25 ± 1	2 ± 1	27 ± 4
23 ± 5	32 ± 1	43 ± 9	40 ± 2	65 ± 5	56 ± 0
67 ±14	52 ± 4	58 ± 8	68 ± 1	66 ±14	49 ± 1
71 ± 2	75 ± 7	28 ± 5	62 ± 7	65 ±11	54 ± 1
106 ± 4	67 ± 4	91 ± 0	70 ± 0	98 ± 3	72 ± 1

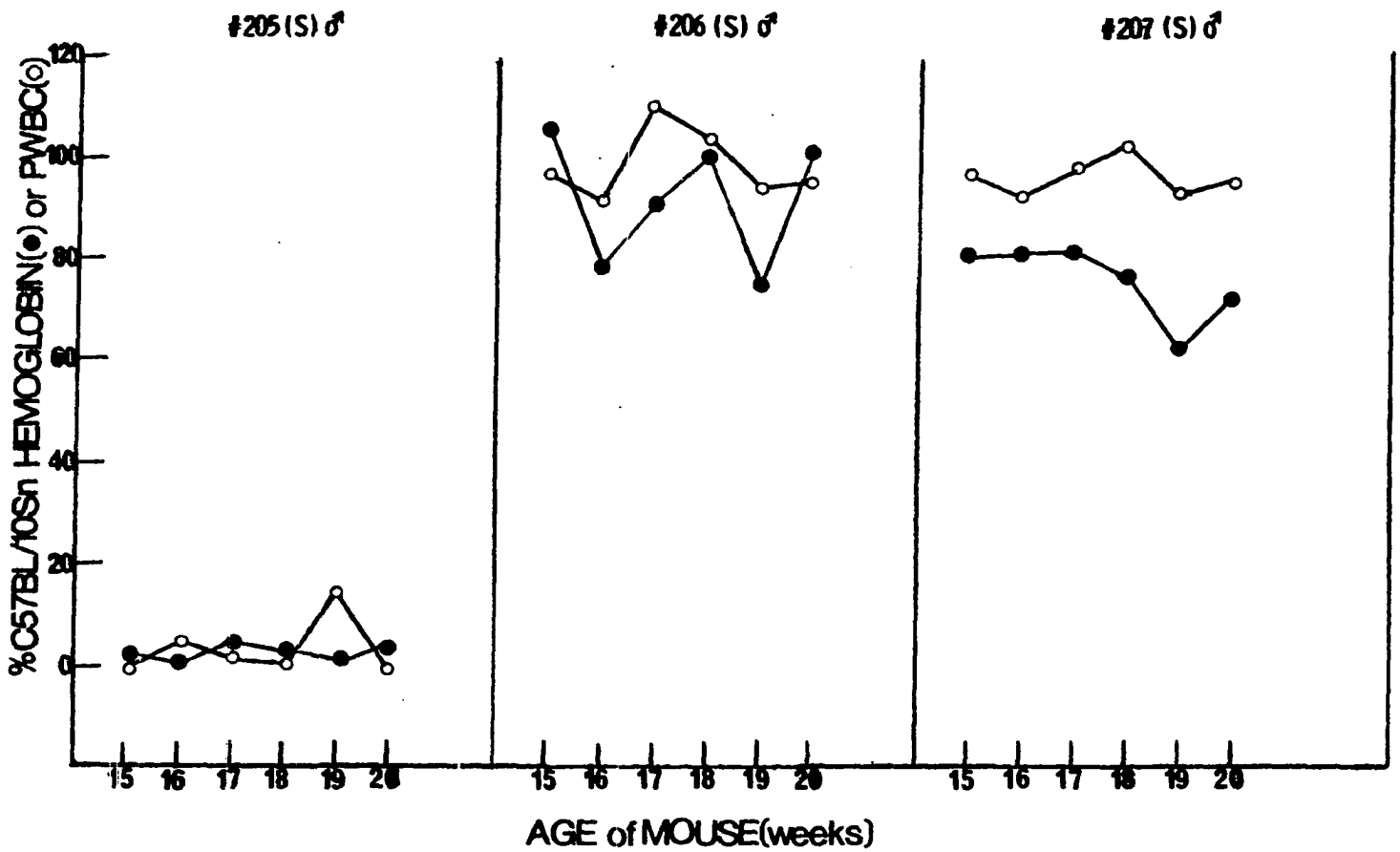


Figure 25. Hemoglobin and peripheral white blood cell (PWBC) composition of C57BL/10Sn ⟷ A mice as a function of age of mice. [S and M refer to single- and multicolored mice, respectively.]

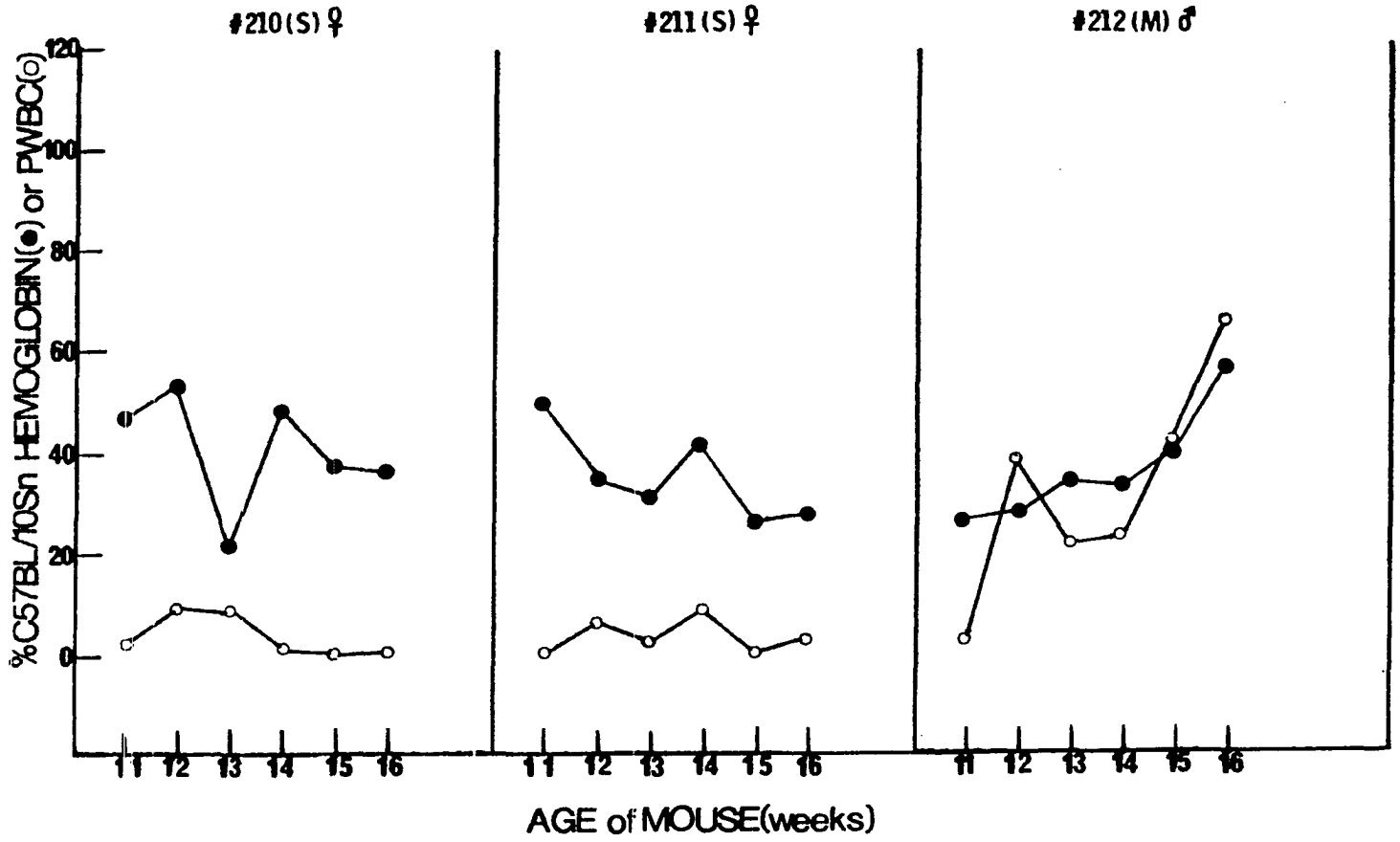


Figure 25. (continued)

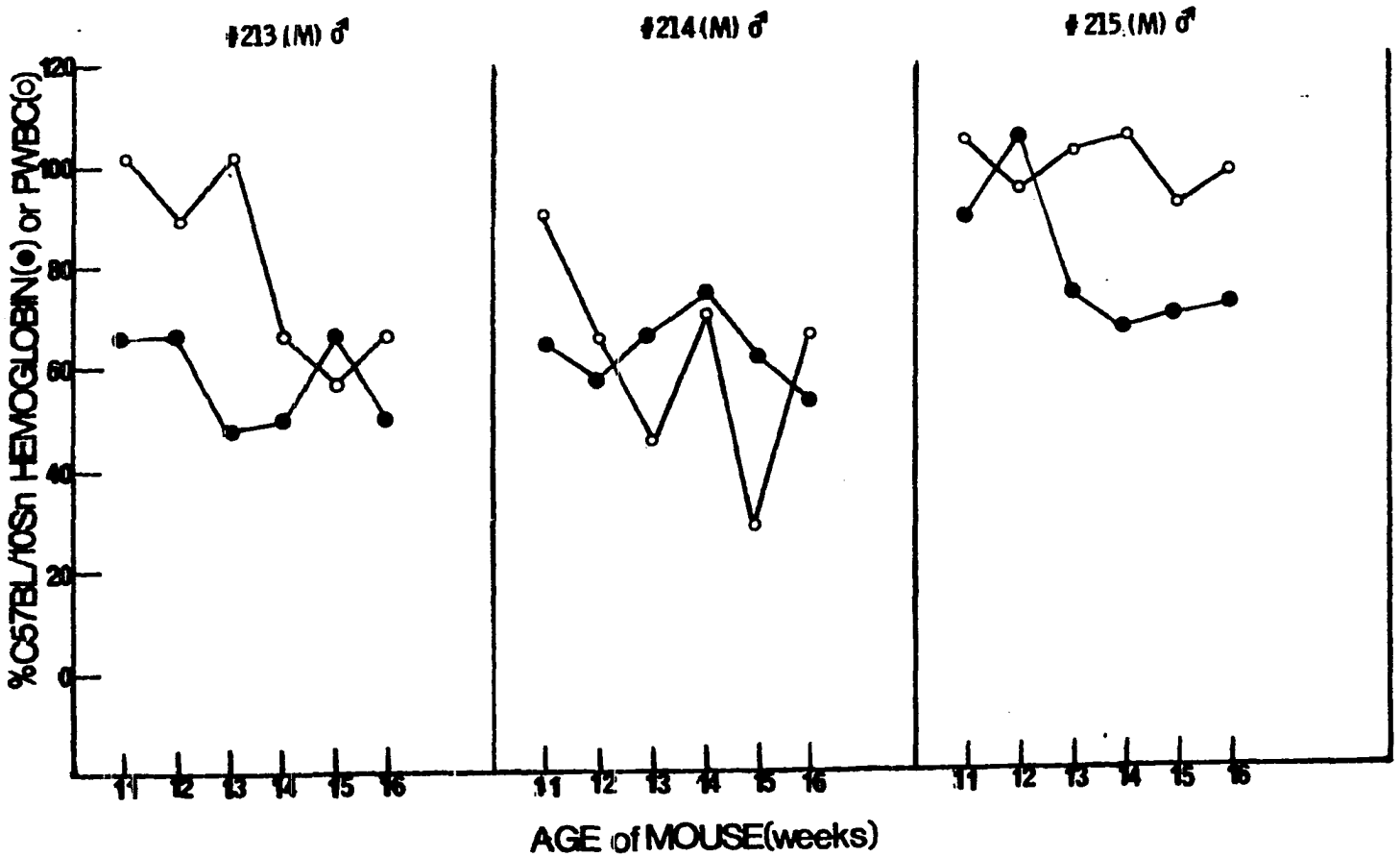


Figure 25. (continued)

Table 18. Analysis of C57BL/10Sn ⟷ A allophenic mice

Mouse Number	Percentage of C57BL/10Sn or A PWBC[a]					
	First Determination		Second Determination		Third Determination	
	C57BL/10Sn	A	C57BL/10Sn	A	C57BL/10Sn	A
205	0 ± 0	99 ± 1	6 ± 2	99 ± 1	3 ± 1	104 ± 5
206	97 ± 0	0 ± 0	91 ± 5	2 ± 2	111 ± 0	0
207	97 ± 2	7 ± 1	94 ± 2	2 ± 1	99 ± 2	9 ± 1
210	3 ± 3	100 ± 2	10 ± 7	96 ± 8	9 ± 2	98 ± 3
211	0 ± 0	106 ± 2	6 ± 3	101 ± 2	3 ± 1	102 ± 4
212	2 ± 1	92 ± 1	38 ± 5	80 ± 2	21 ± 6	74 ± 1
213	103 ± 5	18 ± 0	90 ± 2	21 ± 1	103 ± 2	5 ± 2
214	91 ± 5	24 ± 5	67 ± 4	27 ± 4	45 ± 0	56 ± 3
215	105 ± 3	1 ± 0	94 ± 3	1 ± 1	103 ± 6	8 ± 1

[a]The percentage of C57BL/10Sn or A peripheral white blood cells (PWBC) was determined as described in the Materials and Methods section.

Percentage of C57BL/10Sn or A PWBC[a]					
Fourth Determination		Fifth Determination		Sixth Determination	
C57BL/10Sn	A	C57BL/10Sn	A	C57BL/10Sn	A
2 ± 5	105 ± 2	15 ± 6	78 ± 0	0 ± 0	101 ± 2
104 ± 0	2 ± 1	95 ± 1	8 ± 4	100 ± 2	3 ± 1
103 ± 1	4 ± 1	94 ± 1	4 ± 1	96 ± 2	6 ± 1
2 ± 1	104 ± 2	0 ± 0	95 ± 0	1 ± 1	92 ± 7
9 ± 8	99 ± 5	0 ± 0	97 ± 6	2 ± 1	98 ± 3
23 ± 5	87 ± 6	43 ± 0	61 ± 10	65 ± 5	56 ± 4
67 ± 14	44 ± 0	58 ± 8	68 ± 7	66 ± 14	52 ± 6
71 ± 2	53 ± 0	28 ± 5	74 ± 2	65 ± 11	41 ± 2
106 ± 4	15 ± 3	91 ± 0	7 ± 1	98 ± 3	1 ± 1

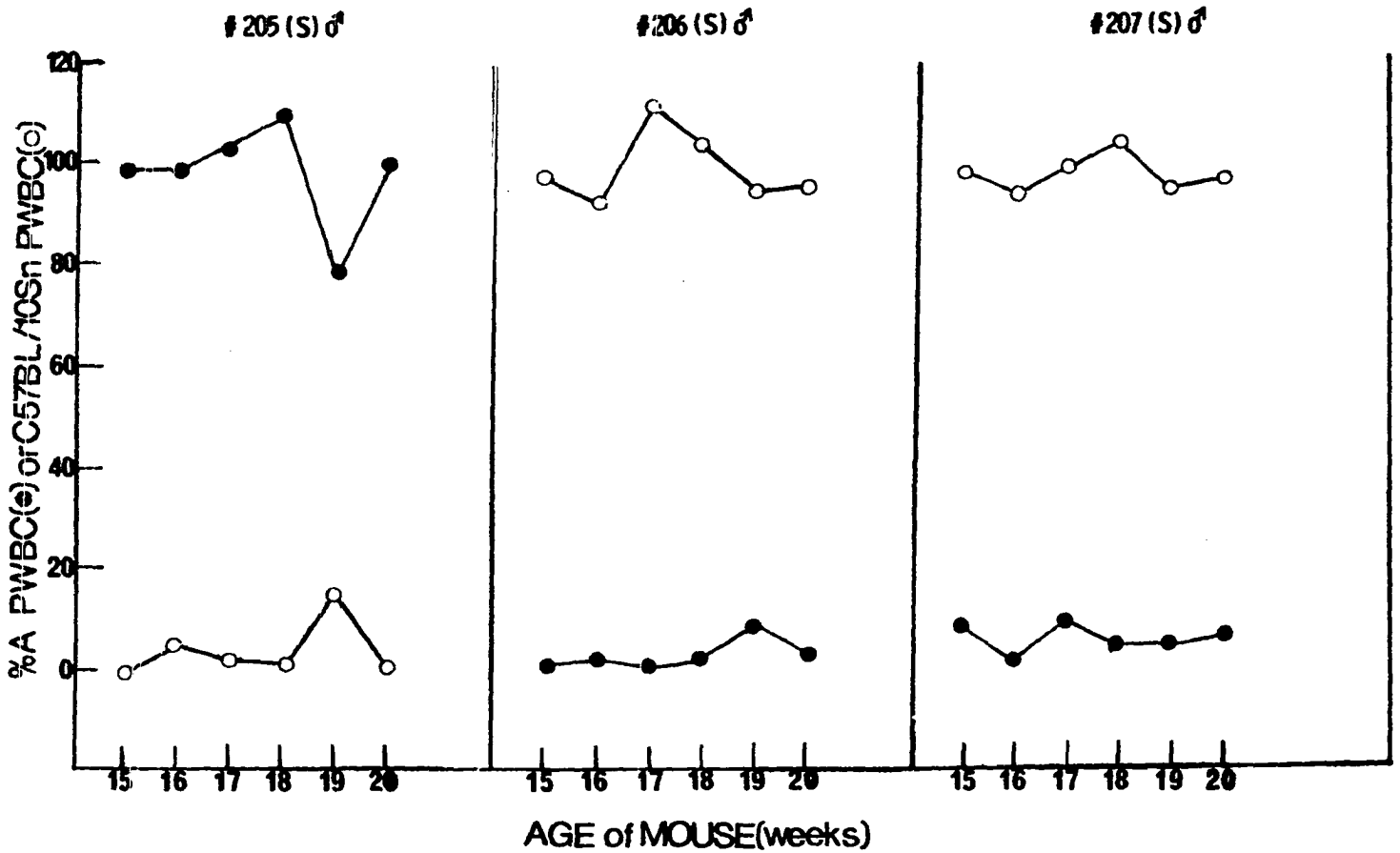


Figure 26. Peripheral white blood cell (PWBC) composition of C57BL/10Sn ⟷ A mice as a function of age of mice. S and M refer to single- and multicolored mice, respectively.

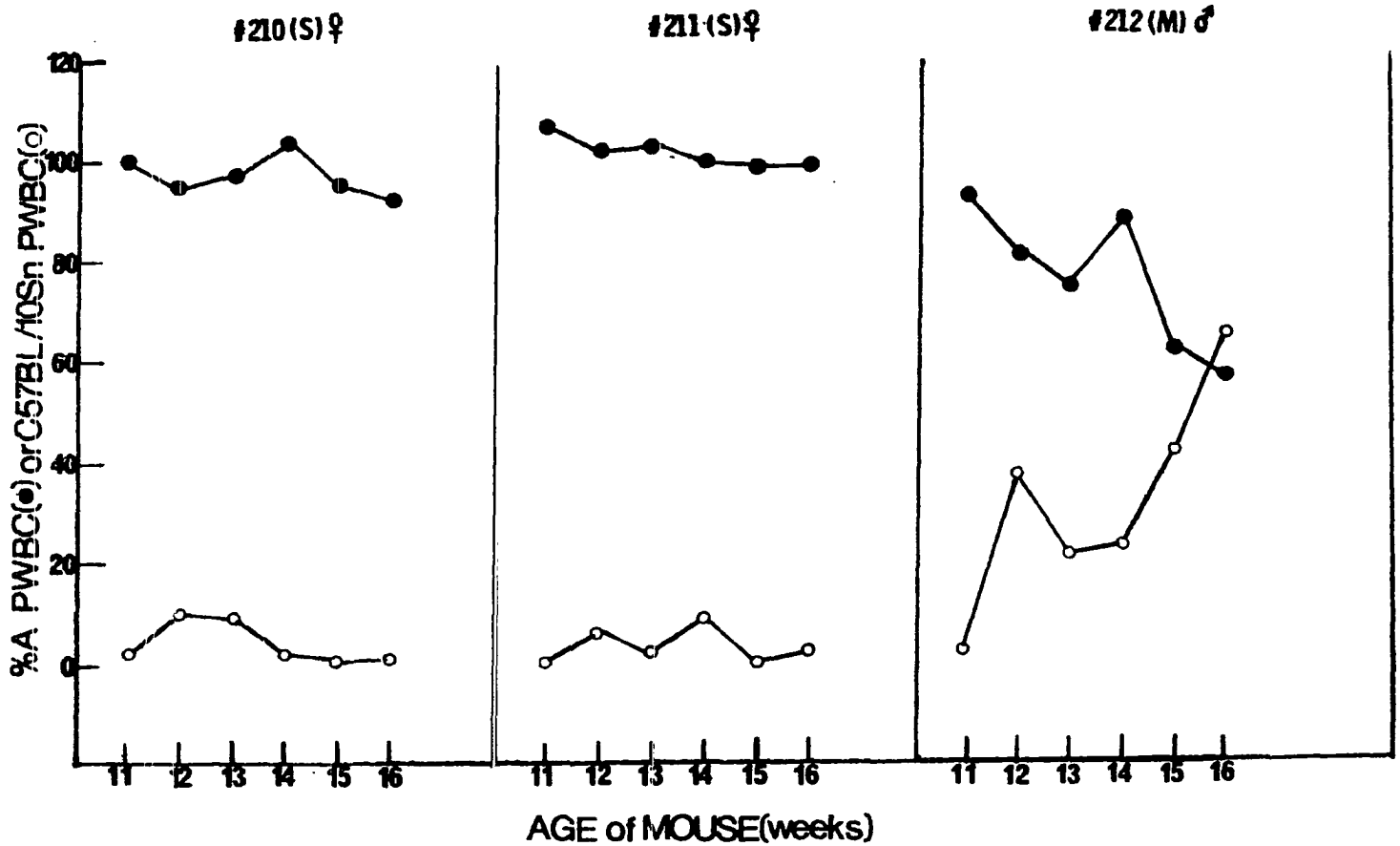


Figure 26. (continued)

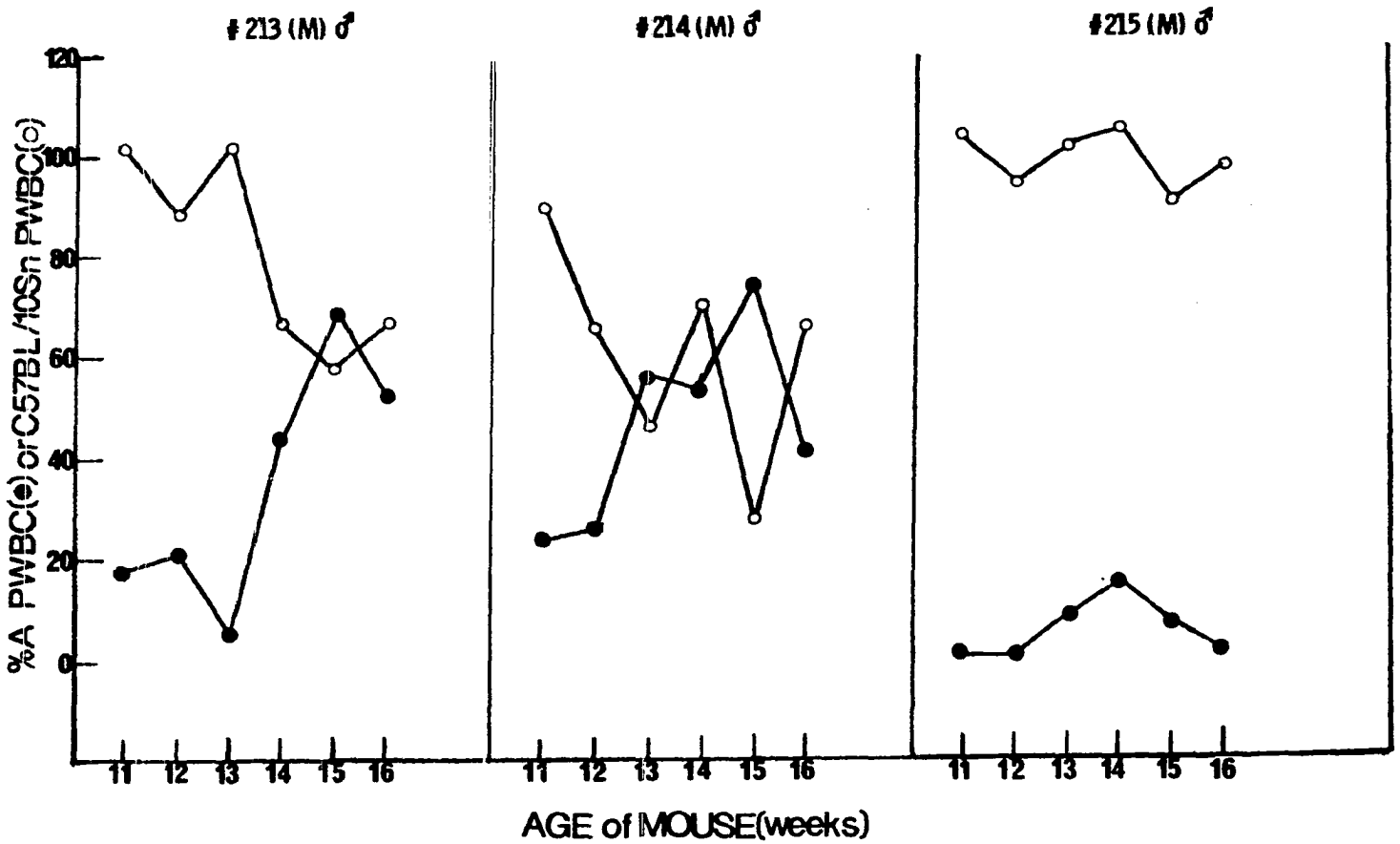


Figure 26. (continued)

of from two to five months apart. In the second set, hemoglobin composition was tested monthly for six to eight months, and the peripheral white blood cell composition was tested three times at two-month intervals. It is seen from Figures 21, 22, and 23 that the allophenic mice were between two and sixteen months of age for all of the determinations.

The peripheral white blood cell and hemoglobin compositions of 15 C57BL/6 ⟷ A mice as shown in Figure 24 were measured at either two or three months of age (first determination), one month later (second determination) and then three months after that (third determination). For all three determinations, the peripheral white blood cell and hemoglobin were collected within one day of each other.

Data shown in Figure 25 summarizes the peripheral white blood cell and hemoglobin composition of 9 C57BL/10Sn ⟷ A mice. Six weekly samples were collected from mice either 11 or 15 weeks of age at the beginning of the experiment. Data presented in Table 18 and Figure 26 show in detail the changes in both parental types peripheral white blood cells during the same time period. Almost all of the data presented in Tables 13, 14, 15, 16, 17, 18, and Figures 21, 22, 23, 24, 25, and 26 are the average of duplicate determinations. The number of each mouse, its sex, and whether or not it was single (S)-colored or multi (M)-colored are shown in each graph.

Data are shown in Table 19 for the analysis of the parental types peripheral white blood cells at time 0 (two months before sacrifice) and time 0+2 (time of sacrifice) for 33 allophenic mice. The mice included

Table 19. Analysis of peripheral white blood cell changes in allophenic mice[a]

Mouse Number	Sex	Coat Color Phenotype[b] %C57BL/6	Coat Color Phenotype[b] %A x SJL	Age at Sacrifice (Months)	Peripheral White Blood Cells-Time 0[c] %C57BL/6	Peripheral White Blood Cells-Time 0[c] %A x SJL	Peripheral White Blood Cells-Time (0+2) %C57BL/6	Peripheral White Blood Cells-Time (0+2) %A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1								
133	♂	0	100	11	1 ± 0	109 ± 1	1 ± 0	91 ± 4
134	♀	0	100	11	3 ± 1	100 ± 3	3 ± 2	93 ± 3
135	♀	0	100	11	0 ± 0	105 ± 3	2 ± 1	89 ± 4
137	♀	0	100	9	2 ± 1	100 ± 0	1 ± 1	96 ± 3
138	♀	15	85	9	2 ± 1	73 ± 1	8 ± 2	110 ± 5
139	♂	20	80	9	1 ± 1	70 ± 3	6 ± 5	74 ± 4
140	♀	25	75	9	0 ± 0	82 ± 5	2 ± 2	98 ± 3
141	♀	30	70	9	0 ± 0	61 ± 4	1 ± 0	120 ± 20
142	♂	85	15	9	7 ± 6	57 ± 4	23 ± 1	73 ± 1
143	♂	95	5	9	19 ± 2	70 ± 1	24 ± 5	80 ± 1
148	♀	0	100	11	5 ± 1	102 ± 3	11 ± 2	96 ± 2
149	♂	0	100	11	5 ± 0	100 ± 0	11 ± 3	98 ± 0
150	♀	0	100	11	3 ± 1	94 ± 1	9 ± 5	107 ± 2
151	♂	5	95	11	7 ± 1	97 ± 2	4 ± 5	107 ± 2
152	♀	15	85	11	4 ± 3	97 ± 3	6 ± 0	97 ± 1
153	♀	80	20	11	22 ± 1	71 ± 4	10 ± 1	103 ± 5

[a] All values are the average of duplicate determinations.

[b] Coat color was estimated visually at the time of weaning.

[c] Time 0 is two months before sacrifice. Time (0+2) is the time of sacrifice.

Table 19. (continued)

Mouse Number	Sex	Coat Color Phenotype[b] %CBA[d]	%DBA/1	Age at Sacrifice (Months)	Peripheral White Blood Cells-Time 0[c] %CBA[d]	%DBA/1	Peripheral White Blood Cells-Time (0+2) % CBA[d]	% DBA/1
B. (CBA x CBA/H-T6)F_1 ⟷ DBA/1								
144	♂	50	50	13	--	32 ± 2	--	62 ± 1
145	♂	100	0	13	--	3 ± 0	--	3 ± 0
146	♂	100	0	13	--	14 ± 3	--	3 ± 0
154	♂	60	40	10	--	17 ± 1	--	6 ± 0
163	♀	0	100	9	--	100 ± 2	--	93 ± 0
164	♀	5	95	9	--	64 ± 4	--	67 ± 0
165	♂	50	50	9	--	2 ± 2	--	36 ± 5
166	♂	40	60	9	--	20 ± 2	--	38 ± 3
		%CBA[d]	%C57BL/6		%CBA[d]	%C57BL/6	%CBA[d]	%C57BL/6
C. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6								
125	♂	15	85	11	0 ± 0	74 ± 2	1 ± 0	83 ± 4
126	♂	60	40	11	42 ± 2	48 ± 1	58 ± 5	72 ± 1
127	♂	90	10	11	19 ± 1	69 ± 2	17 ± 1	69 ± 7
128	♂	95	5	11	20 ± 3	31 ± 1	32 ±3	52 ± 2
129	♀	98	2	11	95 ± 1	20 ± 3	67 ± 4	19 ± 0
130	♂	100	0	11	108 ± 2	0 ± 1	78 ± 3	0 ± 0
131	♂	100	0	11	105 ± 3	1 ± 0	100 ± 0	1 ± 2

[d]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 19. (continued)

Mouse Number	Sex	Coat Color Phenotype[b] %C57BL/6	%DBA/1	Age at Sacrifice (Months)	Peripheral White Blood Cells-Time 0[c] %C57BL/6	%DBA/1	Peripheral White Blood Cells-Time (0+2) %C57BL/6	%DBA/1
D. C57BL/6 ⟷ DBA/1								
123	♂	100	0	16	34 ± 1	2 ± 2	79 ± 2	15 ± 3
124	♂	50	50	16	43 ± 2	18 ± 2	69 ± 4	39 ± 1

16 C57BL/6 ⟷ (A x SJL)F_1 (10 mice previously characterized in Table 13, plus 6 additional mice), 8 (CBA x CBA/H-T6)F_1 ⟷ DBA/1, 7 (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 (all mice previously characterized in Table 14). Of the mice, 20 were phenotypic males and 13 were phenotypic females. In addition, 20 of the mice showed both parental coat colors, which 13 of the mice showing one or the other parental coat color. All the experimental values in Table 19 are the average of duplicate determinations. Changes in hemoglobin or peripheral white blood cell composition with time have been called chimeric drift. A summary of the mice which have shown chimeric drift is shown in Table 20.

Table 21 summarizes the statistical analysis of the correlation of hemoglobin and peripheral white blood cell composition of allophenic mice at all time points. Data were taken from Tables 13, 14, 15, 16, and 17 for Table 21. Unstable mice refers to allophenic mice showing marked changes in their red blood cell or white blood cell populations with time. Stable mice show no change. Figure 27 shows a plot of the hemoglobin composition of 15 C57BL/6 ⟷ A mice as a function of their peripheral white blood cell composition at any given time point. Data for Figure 27 were taken from Table 16.

G. Composition of the Immune System of Allophenic Mice

The characterization of the 93 allophenic mice used in this study is shown in Table 22. The mice included 34 C57BL/6 ⟷ (A x SJL)F_1 mice, 3 DBA/1 ⟷ (A x SJL)F_1 mice, 3 DBA/1 ⟷ A

Table 20. List of allophenic mice showing chimeric drift

Mouse Type	Number Mice Tested		Hemoglobin Drift[a]		PWBC Drift[b]	
	Hemoglobin	PWBC	Total Number Mice	Mouse Number	Total Number Mice	Mouse Number
A. C57BL/6 ↔ (A x SJL)F_1	28	34	5	84, 85, 114, 137, 143	3	83, 84, 141
B. (CBA x CBA/H-T6)F_1 ↔ DBA/1	0	8	--	--	0	--
C. (CBA x CBA/H-T6)F_1 ↔ C57BL/6	15	15	2	76, 119	1	128
D. C57BL/6 ↔ DBA/1	4	4	0	--	1	123
E. C57BL/6 ↔ A	15	15	2	159, 191	5	158, 162, 173 187, 188
F. C57BL/10Sn ↔ A	9	9	0	--	3	212, 213, 214

[a] A significant change in hemoglobin content was considered to be ≥ 40% of total.

[b] A significant change in PWBC content was considered to be ≥ 40% of total.

Table 21. Statistical analysis of the correlation of hemoglobin and PWBC composition of allophenic mice

Mouse Type	Number Data Points	Correlation Coefficient	Probability[a]
A. C57BL/6 ↔ (A x SJL)F_1			
All Mice	66	0.82	<0.001
Stable Mice	52	0.87	<0.001
Unstable Mice	14	0.56	<0.05
B. (CBA x CBA/H-T6)F_1 ↔ C57BL/6			
All Mice	37	0.77	<0.001
Stable Mice	30	0.83	<0.001
Unstable Mice	7	0.12	>0.1
C. C57BL/6 ↔ DBA/1			
All Mice	9	0.75	<0.05
Stable Mice	6	0.95	<0.01
Unstable Mice	3	-0.80	>0.1
D. C57BL/6 ↔ A			
All Mice	45	0.80	<0.001
Stable Mice	24	0.94	<0.001
Unstable Mice	21	0.58	<0.01
E. C57BL/10Sn ↔ A			
All Mice	54	0.84	<0.001
Stable Mice	36	0.88	<0.001
Unstable Mice	18	0.64	<0.01

[a]This is the probability that the variables are not correlated, as determined by Bevington (1969).

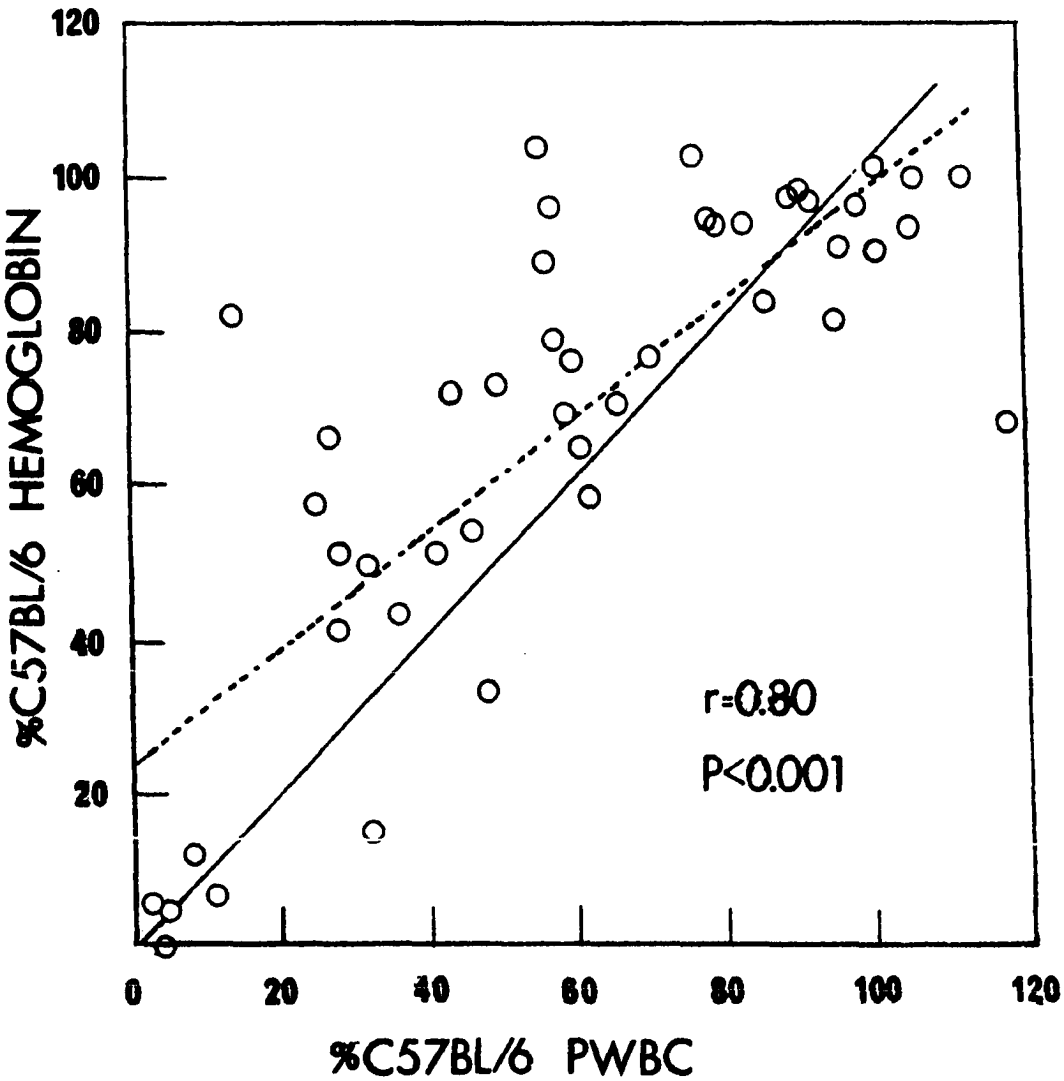


Figure 27. Percent C57BL/6 hemoglobin as a function of the percent C57BL/6 peripheral white blood cells (PWBC). [(——) is the theoretical line for a perfect correlation; (---) is the least squares line through the points.]

Table 22. Composition of the immune system of allophenic mice at time of sacrifice[a]

Mouse Number	Sex	Coat Color Phenotype[b] %C57BL/6	Coat Color Phenotype[b] %(A x SJL)F_1	Age at Sacrifice (Months)
A. C57BL/6 ⟷ (A x SJL)F_1				
83	♂	75	25	12
84	♂	40	60	12
85	♀	85	15	12
86	♂	10	90	11
87	♀	15	85	11
98	♂	0	100	10
108	♂	50	50	10
109	♂	0	100	10
110	♀	100	0	10
111	♀	0	100	10
112	♂	90	10	10
113	♀	0	100	10
114	♀	0	100	10
115	♀	100	0	10
116	♂	50	50	10
117	♂	0	100	10
118	♀	100	0	10
132	♀	0	100	11
133	♂	0	100	11
134	♀	0	100	11
135	♀	0	100	11
137	♀	0	100	9
138	♀	15	85	9
139	♂	20	80	9
140	♀	25	75	9

[a]All values are the average of duplicate determinations.

[b]Coat color phenotype was estimated visually at the time of weaning.

Peripheral White Blood Cells		Spleen White Blood Cells		Thymocytes	
%C57BL/6	%(A x SJL)F_1	%C57BL/6	%(A x SJL)F_1	%C57BL/6	%(A x SJL)F_1
10 ± 1	94 ± 1	7 ± 3	87 ± 9	--	--
16 ± 2	105 ± 15	13 ± 2	85 ± 1	--	--
53 ± 4	71 ± 20	3 ± 1	100 ± 3	--	--
6 ± 1	92 ± 1	3 ± 0	106 ± 1	--	--
46 ± 2	86 ± 3	25 ± 2	48 ± 1	--	--
4 ± 1	88 ± 5	4 ± 1	91 ± 7	--	--
7 ± 3	59 ± 5	11 ± 1	82 ± 0	--	--
8 ± 1	90 ± 0	3 ± 8	97 ± 1	--	--
71 ± 0	21 ± 1	99 ± 3	6 ± 1	--	--
11 ± 5	98 ± 0	2 ± 2	97 ± 1	--	--
14 ± 3	68 ± 0	25 ± 2	66 ± 5	--	--
13 ± 2	105 ± 1	2 ± 3	111 ± 3	--	--
8 ± 5	84 ± 1	2 ± 1	100 ± 3	--	--
85 ± 0	6 ± 2	102 ± 1	5 ± 0	--	--
12 ± 3	88 ± 1	1 ± 1	87 ± 5	--	--
16 ± 2	67 ± 5	4 ± 0	100 ± 9	--	--
98 ± 9	7 ± 2	100 ± 9	10 ± 2	--	--
--	--	--	--	--	--
1 ± 0	91 ± 4	2 ± 0	112 ± 3	--	99 ± 8
3 ± 2	93 ± 3	2 ± 0	106 ± 4	--	92 ± 0
2 ± 1	89 ± 4	2 ± 0	106 ± 0	--	97 ± 3
1 ± 1	96 ± 3	5 ± 1	89 ± 0	--	105 ± 0
8 ± 2	110 ± 5	3 ± 0	101 ± 1	--	94 ± 5
6 ± 5	74 ± 4	1 ± 1	96 ± 3	--	93 ± 2
2 ± 2	98 ± 3	3 ± 3	93 ± 2	--	105 ± 1

Table 22. (continued)

Mouse Number	Sex	Coat Color Phenotype[b] %C57BL/6	%(A x SJL)F_1	Age at Sacrifice (Months)
141	♀	30	70	9
142	♂	85	15	9
143	♂	95	5	9
148	♀	0	100	11
149	♂	0	100	11
150	♀	0	100	11
151	♂	5	95	11
152	♀	15	85	11
153	♀	80	20	11
		%DBA/1	%(A x SJL)F_1	
B. DBA/1 ⟷ (A x SJL)F_1				
94	♂	30	70	10
99	♂	60	40	10
100	♂	80	20	10
		% DBA/1	%A	
C. DBA/1 ⟷ A				
182	♂	75	25	12
183	♂	100	0	12
186	♂	98	2	12
		%CBA[c]	%DBA/1	
D. (CBA x CBA/H-T6)F_1 ⟷ DBA/1				
97	♀	5	95	10
103	♂	100	0	10
104	♂	50	50	10
105	♂	50	50	10
106	♀	0	100	10
107	♂	0	100	10
144	♂	50	50	13
145	♂	100	0	13
146	♂	100	0	13
154	♂	60	40	10

[c] CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Peripheral White Blood Cells		Spleen White Blood Cells		Thymocytes	
%C57BL/6	%(A x SJL)F_1	%C57BL/6	%(A x SJL)F_1	%C57BL/6	%(A x SJL)F_1
1 ± 0	120 ± 20	11 ± 1	102 ± 2	--	100 ± 15
23 ± 1	73 ± 1	14 ± 1	86 ± 1	--	71 ± 9
24 ± 5	80 ± 1	23 ± 5	61 ± 1	--	64 ± 4
11 ± 2	96 ± 2	2 ± 2	102 ± 2	5 ± 2	90 ± 3
11 ± 3	98 ± 0	4 ± 1	102 ± 1	7 ± 2	92 ± 7
9 ± 5	107 ± 2	8 ± 1	102 ± 2	0 ± 0	97 ± 2
4 ± 5	107 ± 2	9 ± 5	103 ± 15	0 ± 0	99 ± 1
6 ± 0	97 ± 1	6 ± 0	107 ± 0	0 ± 0	95 ± 1
10 ± 1	103 ± 5	9 ± 5	95 ± 2	49 ± 4	46 ± 2
%DBA/1	%(A x SJL)F_1	%DBA/1	%(A x SJL)F_1	%DBA/1	%(A x SJL)F_1
22 ± 1	--	7 ± 2	--	--	--
41 ± 1	--	14 ± 2	--	--	--
62 ± 0	--	16 ± 1	--	--	--
%DBA/1	%A	%DBA/1	%A	%DBA/1	%A
44 ± 1	89 ± 1	35 ± 1	87 ± 0	15 ± 0	91 ± 1
67 ± 2	18 ± 3	93 ± 1	16 ± 0	110 ± 1	7 ± 1
67 ± 1	18 ± 2	95 ± 1	15 ± 0	86 ± 0	18 ± 13
%CBA[c]	%DBA/1	%CBA[c]	%DBA/1	%CBA[c]	%DBA/1
--	--	33 ± 2	74 ± 6	--	--
92 ± 1	9 ± 0	89 ± 2	4 ± 1	--	--
85 ± 0	27 ± 2	70 ± 2	22 ± 2	--	--
83 ± 4	43 ± 2	54 ± 1	48 ± 2	--	--
8 ± 3	92 ± 0	2 ± 1	102 ± 1	--	--
20 ± 4	64 ± 4	16 ± 4	85 ± 2	--	--
--	62 ± 1	--	60 ± 1	--	95 ± 4
--	3 ± 0	--	2 ± 0	--	3 ± 5
--	3 ± 0	100 ± 1	2 ± 0	--	3 ± 2
--	6 ± 0	104 ± 2	7 ± 2	--	8 ± 3

Table 22. (continued)

Mouse Number	Sex	Coat Color Phenotype[b] %CBA[c]	%DBA/1	Age at Sacrifice (Months)
163	♀	0	100	9
164	♀	5	95	9
165	♂	50	50	9
166	♂	40	60	9
174	♂	100	0	9
175	♀	40	60	9
		%CBA[c]	%C57BL/6	
E. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6				
74	♀	0	100	14
76	♀	20	80	14
80	♂	0	100	14
81	♂	0	100	14
82	♂	20	80	14
119	♀	10	90	9
120	♀	100	0	9
121	♂	95	5	9
125	♂	15	85	12
126	♂	60	40	12
127	♂	90	10	12
128	♂	95	5	12
129	♀	98	2	12
130	♂	100	0	12
131	♂	100	0	12
184	♂	98	2	10
		%CBA[c]	%A	
F. (CBA x CBA/H-T6)F_1 ⟷ A				
177	♂	35	65	10
178	♂	40	60	10
179	♂	100	--	10
180	♂	100	--	10
181	♀	100	--	10

Peripheral White Blood Cells		Spleen White Blood Cells		Thymocytes	
%CBA[c]	%DBA/1	%CBA[c]	%DBA/1	%CBA[c]	%DBA/1
--	93 ± 0	7 ± 1	104 ± 0	--	84 ± 2
--	67 ± 0	16 ± 2	93 ± 0	--	109 ± 5
--	35 ± 5	106 ± 1	8 ± 1	--	25 ± 4
--	38 ± 3	81 ± 0	40 ± 3	--	0 ± 0
106 ± 0	0 ± 0	101 ± 0	7 ± 1	94 ± 0	0 ± 0
4	107	4	83	24	75

%CBA[c]	%C57BL/6	%CBA[c]	%C57BL/6	%CBA[c]	%C57BL/6
7 ± 1	99 ± 2	8 ± 2	94 ± 2	--	--
27 ± 0	85 ± 4	12 ± 4	80 ± 3	--	--
11 ± 1	103 ± 3	4 ± 1	94 ± 0	--	--
5 ± 1	105 ± 5	5 ± 2	101 ± 5	--	--
33 ± 0	61 ± 1	22 ± 2	90 ± 1	--	--
33 ± 3	66 ± 2	18 ± 3	58 ± 2	--	--
70 ± 2	41 ± 3	83 ± 3	16 ± 3	--	--
46 ± 4	62 ± 2	32 ± 1	62 ± 1	--	--
1 ± 0	83 ± 4	17 ± 1	86 ± 3	25 ± 10	--
58 ± 5	72 ± 1	58 ± 2	52 ± 0	26 ± 0	--
17 ± 1	69 ± 7	32 ± 2	69 ± 1	17 ± 3	--
32 ± 3	52 ± 2	51 ± 1	55 ± 0	19 ± 4	--
67 ± 4	19 ± 0	61 ± 3	26 ± 0	45 ± 2	--
78 ± 3	0 ± 0	102 ± 1	5 ± 5	71 ± 13	--
100 ± 0	1 ± 2	102 ± 1	3 ± 0	104 ± 10	--
13 ± 1	--	56 ± 3	--	38 ± 2	65 ± 1

%CBA[c]	%A	%CBA[c]	%A	%CBA[c]	%A
15 ± 0	--	60 ± 4	--	--	--
42 ± 2	--	21 ± 0	--	--	--
100 ± 0	--	104 ± 1	--	--	--
98 ± 2	--	104 ± 1	--	--	--
95 ± 1	--	100 ± 2	--	--	--

Table 22. (continued)

Mouse Number	Sex	Coat Color Phenotype[b] %C57BL/6	%DBA/1	Age at Sacrifice (Months)
G. C57BL/6 ⟷ DBA/1				
101	♂	0	100	10
123	♂	100	0	16
124	♂	50	50	16
		%C57BL/6	%A	
H. C57BL/6 ⟷ A				
157	♂	2	98	10
158	♀	95	5	10
159	♀	40	60	10
160	♂	100	0	9
162	♂	50	50	9
167	♂	100	0	9
168	♂	85	15	9
170	♀	0	100	9
171	♂	50	50	9
172	♀	60	40	9
173	♂	75	25	9
187	♀	100	0	8
191	♀	50	50	8

Peripheral White Blood Cells		Spleen White Blood Cells		Thymocytes	
%C57BL/6	%DBA/1	%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
13 ± 0	100 ± 1	14	100 ± 1	--	--
79 ± 2	15 ± 3	69 ± 2	7 ± 0	--	85 ± 1
69 ± 4	39 ± 1	42 ± 0	35 ± 0	--	71 ± 1
%C57BL/6	%A	%C57BL/6	%A	%C57BL/6	%A
100 ± 0	0 ± 0	95 ± 0	16 ± 2	90 ± 16	5 ± 1
98 ± 3	11 ± 2	97 ± 2	20 ± 0	107 ± 4	0 ± 0
16 ± 2	68 ± 4	11 ± 1	85 ± 5	57 ± 2	110 ± 2
98 ± 2	0 ± 0	72 ± 5	26 ± 1	111 ± 2	0 ± 0
54 ± 2	39 ± 3	63 ± 4	46 ± 0	73 ± 4	47 ± 0
79 ± 1	15 ± 1	106 ± 1	13 ± 2	102 ± 1	8 ± 0
91 ± 1	12 ± 3	95 ± 3	29 ± 4	93 ± 3	13 ± 2
0 ± 0	109 ± 4	1 ± 0	99 ± 0	2 ± 0	100 ± 1
3 ± 3	97 ± 1	3 ± 1	98 ± 1	1 ± 1	97 ± 1
76 ± 1	32 ± 2	52 ± 1	61 ± 1	71 ± 4	30 ± 2
71 ± 1	44 ± 2	59 ± 1	55 ± 0	32 ± 2	73 ± 2
103 ± 0	2 ± 3	84 ± 1	8 ± 1	105 ± 0	0 ± 0
35 ± 2	60 ± 1	12 ± 2	92 ± 0	12 ± 2	96 ± 2

mice, 16 (CBA x CBA/H-T6)F_1 ⟷ DBA/1 mice, 16 (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 mice, 5 (CBA x CBA/H-T6)F_1 ⟷ A mice, 3 C57BL/6 ⟷ DBA/1 mice, and 13 C57BL/6 ⟷ A mice. Of the 93 mice, 57 were phenotypic males and 36 were phenotypic females. In addition, 53 of the mice showed both parental coat colors, while 40 of the mice showed one or the other parental coat color. Data are shown in Table 22 for the analysis of the parental types of peripheral white blood cells, spleen white blood cells and thymocytes (when possible) at the time of sacrifice. All the experimental values in Table 22 are the average of duplicate determinations. Table 23 shows the additivity of peripheral white blood cells, spleen white blood cells, and thymocytes of various combinations of allophenic mice. The data used for these calculations are from Table 22. Table 24 shows the correlation of the various cell types determined at the time of sacrifice.

Differences between the relative proportion of the parental white blood cells in the spleen and thymus has been called spleen-thymus discordance. A summary of the mice which have shown chimeric drift and spleen-thymus discordance is shown in Table 25. The data for chimeric drift and spleen-thymus discordance were taken from Tables 13, 14, 15, 16, 17, 18, 19, and 22, respectively.

H. Analysis of Chimeric Serum Blocking Factors

1. Allophenic mouse sera and allophenic mouse spleen white blood cells

In Tables 26 and 27 are shown the results of 26 allophenic mouse serum samples examined for serum blocking activity using the trypan blue dye

Table 23. Additivity of cell types in allophenic mice[a]

Mouse Type	Number of Mice	Mean ± S.D.		
		Peripheral White Blood Cells	Spleen White Blood Cells	Thymocytes
A. C57BL/6 ⟷ (A x SJL)F_1	34	102 ± 14	102 ± 8	97 ± 2
B. DBA/1 ⟷ (A x SJL)F_1	3	--	--	--
C. DBA/1 ⟷ A	3	101 ± 23	107 ± 5	109 ± 6
D. (CBA x CBA/H-T6)F_1 ⟷ DBA/1	16	107 ± 13	104 ± 10	97 ± 3
E. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6	16	101 ± 16	101 ± 9	103
F. (CBA x CBA/H-T6)F_1 ⟷ A	5	--	--	--
G. C57BL/6 ⟷ DBA/1	3	105 ± 8	89 ± 18	--
H. C57BL/6 ⟷ A	13	101 ± 8	108 ± 9	110 ± 18

[a]The data used for these calculations were taken from Table 22.

Table 24. Correlation analysis of cell types in allophenic mice[a]

Comparison of Cell Types[c]	Parental Cell Types[b]					
	C57BL/6			A x SJL		
	N	r	P	N	r	P
A. C57BL/6 ⟷ (A x SJL)F_1						
SWBC versus PWBC	33	0.90	<0.001	33	0.83	<0.001
SWBC versus TWBC	6	0.35	>0.1	16	0.52	<0.05
PWBC versus TWBC	6	0.35	>0.1	16	0.29	>0.1
	DBA/1			(A x SJL)F_1		
	N	r	P	N	r	P
B. DBA/1 ⟷ (A x SJL)F_1						
SWBC versus PWBC	3	0.95	>0.1	--	--	--
SWBC versus TWBC	--	--	--	--	--	--
PWBC versus TWBC	--	--	--	--	--	--
	DBA/1			A		
	N	r	P	N	r	P
C. DBA/1 ⟷ A						
SWBC versus PWBC	3	0.99	<0.1	3	0.99	<0.1
SWBC versus TWBC	3	0.99	<0.1	3	0.99	<0.1
PWBC versus TWBC	3	0.97	>0.1	3	0.99	<0.1

[a]Data taken from Table 22 collected at the time of sacrifice.

[b]Parental cell types refers to the composition of al allophenic mouse as determined by cytotoxicity testing. N = sample size, r = correlation coefficien, P = probability that the variables are not correlated, as determined by Bevington (1969).

[c]Spleen white blood cells (SWBC), peripheral white blood cells (PWBC), and thymocytes (TWBC).

Table 24. (continued)

Comparison of Cell Types[c]	Parental Cell Types[b]					
	CBA[d]			DBA/1		
	N	r	P	N	r	P
D. (CBA x CBA/H-T6)F_1 ⟷ DBA/1						
SWBC versus PWBC	13	0.97	<0.001	15	0.94	<0.001
SWBC versus TWBC	2	1.00	>0.1	10	0.90	<0.001
PWBC versus TWBC	2	1.00	>0.1	10	0.85	<0.001
	CBA[d]			C57BL/6		
	N	r	P	N	r	P
E. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6						
SWBC versus PWBC	16	0.83	<0.001	15	0.93	<0.001
SWBC versus TWBC	8	0.87	<0.01	1	--	--
PWBC versus TWBC	8	0.78	<0.05	1	--	--
	CBA[d]			A		
	N	r	P	N	r	P
F. (CBA x CBA/H-T6)F_1 ⟷ A						
SWBC versus PWBC	5	0.80	>0.1	--	--	--
SWBC versus TWBC	--	--	--	--	--	--
PWBC versus TWBC	--	--	--	--	--	--
	C57BL/6			DBA/1		
	N	r	P	N	r	P
G. C57BL/6 ⟷ DBA/1						
SWBC versus PWBC	3	0.93	>0.1	3	0.99	>0.1
SWBC versus TWBC	--	--	--	2	-1.00	>0.1
PWBC versus TWBC	--	--	--	2	-1.00	>0.1

[d] CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 24. (continued)

Comparison of Cell Types[c]	Parental Cell Types[b]					
	C57BL/6			A		
	N	r	P	N	r	P
H. C57BL/6 ⟷ A						
SWBC versus PWBC	13	0.93	<0.001	13	0.94	<0.001
SWBC versus TWBC	13	0.88	<0.001	13	0.95	<0.001
PWBC versus TWBC	13	0.87	<0.001	13	0.92	<0.001

Table 25. Analysis of chimeric drift and spleen-thymus discordance in allophenic mice

Mouse Number	Chimeric Drift[a]		Spleen-Thymus[b] Discordance
	Hemoglobin	PWBC	
A. C57BL/6 ⟷ (A x SJL)F_1			
83	++	+	NT
84	+++	+++	NT
85	+++	++	NT
86	++	+	NT
87	-	+++	NT
98	-	+++	NT
108	+	++	NT
109	+	+	NT
110	-	+	NT
111	+	+	NT
112	+	+	NT
113	++	-	NT
114	+++	+	NT
115	+	+	NT
116	-	+	NT
117	+	+	NT
118	+	+	NT

[a]Chimeric drift refers to time-dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) <20% change, (++) ≥20% but <40% change, (+++) ≥40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[b]Spleen-thymus discordance refers to the difference between the relative proportions of the parental white blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no change, (NT) not tested. Data were taken from Table 22.

Table 25. (continued)

Mouse Number	Chimeric Drift[a]		Spleen-Thymus[b] Discordance
	Hemoglobin	PWBC	
132	+	+	+
133	+	+	+
134	+	+	+
135	+	+	+
137	+++	+	+
138	+	+	+
139	++	+	+
140	++	+	+
141	+	-	+
142	++	+	+
143	+++	+	+
148	NT	+	+
149	NT	+	+
150	NT	+	+
151	NT	+	+
152	NT	+	+
153	NT	++	+++
B. DBA/1 ⟷ (A x SJL)F_1			
94	NT	NT	NT
99	NT	NT	NT
100	NT	NT	NT
C. DBA/ ⟷ A			
182	NT	NT	+
183	NT	NT	+
186	NT	NT	+

Table 25. (continued)

Mouse Number	Chimeric Drift[a]		Spleen-Thymus[b] Discordance
	Hemoglobin	PWBC	
D. (CBA x CBA/H-T6)F_1 ⟷ DBA/1			
97	NT	NT	NT
103	NT	NT	NT
104	NT	NT	NT
105	NT	NT	NT
106	NT	NT	NT
107	NT	NT	NT
144	NT	++	++
145	NT	-	+
146	NT	+	+
154	NT	+	+
163	NT	+	++
164	NT	+	+
165	NT	++	+
166	NT	+	+++
174	NT	NT	+
175	NT	NT	+
E. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6			
74	-	+	NT
76	+++	+	NT
80	++	++	NT
81	+	+	NT
82	-	+	NT
119	+++	+	NT
120	+	+	NT

Table 25. (continued)

Mouse Number	Chimeric Drift[a]		Spleen-Thymus[b] Discordance
	Hemoglobin	PWBC	
121	+	+	NT
125	+	+	+
126	-	++	++
127	++	++	+
128	+	++	++
129	+	++	+
130	+	++	++
131	+	+	+
184	NT	NT	+
F. (CBA x CBA/H-T6)F_1 ⟷ A			
177	NT	NT	NT
178	NT	NT	NT
179	NT	NT	NT
180	NT	NT	NT
181	NT	NT	NT
G. C57BL/1 ⟷ DBA/1			
101	+	+	NT
122	+	+	NT
123	+	+++	+++
124	+	++	++

Table 25. (continued)

Mouse Number	Chimeric Drift[a]		Spleen-Thymus[b] Discordance
	Hemoglobin	PWBC	
H. C57BL/6 ⟷ A			
157	+	+	+
158	++	+++	+++
159	+++	+	++
160	+	++	++
162	+	+++	+
167	+	++	+
168	+	+	+
169	+	+	NT
170	+	+	+
171	+	+	+
172	++	++	++
173	+	+++	++
187	-	+++	+
188	++	+++	NT
191	+++	+++	+
I. C57BL/10Sn ⟷ A			
205	+	+	NT
206	++	+	NT
207	++	+	NT
210	+	+	NT
211	+	+	NT
212	++	+++	NT
213	+	+++	NT
214	+	+++	NT
215	++	+	NT

Table 26. Analysis of serum blocking factors using allophenic mouse sera and allophenic mouse spleen white blood cells

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	2	0 ± 0	89 ± 4	0 ± 0	76 ± 2*
	4	0 ± 0	84 ± 3	1 ± 1	82 ± 5
	8	0 ± 0	92 ± 1	1 ± 1	82 ± 0
	16	0 ± 0	95 ± 4	1 ± 1	90 ± 1
	32	0 ± 0	90 ± 5	1 ± 1	93 ± 1
134	2	12 ± 1	86 ± 6	5 ± 3*	108 ± 3*
	4	7 ± 0	96 ± 2	3 ± 2	103 ± 5
	8	8 ± 1	102 ± 4	6 ± 0	92 ± 3
135	4	0 ± 0	99 ± 1	1 ± 1	97 ± 1
	8	0 ± 0	93 ± 2	1 ± 1	96 ± 1
	16	0 ± 0	96 ± 6	7 ± 5*	98 ± 0
137	2	2 ± 2	85 ± 4	0 ± 0	87 ± 3
	4	0 ± 0	81 ± 1	0 ± 0	84 ± 3
	8	0 ± 0	82 ± 1	0 ± 0	81 ± 2
	16	0 ± 0	73 ± 3	0 ± 0	91 ± 0**
138	2	0 ± 0	94 ± 2	0 ± 0	96 ± 2
	4	0 ± 0	90 ± 1	0 ± 0	96 ± 1**
	8	0 ± 0	106 ± 1	0 ± 0	98 ± 1**
	16	0 ± 0	100 ± 1	1 ± 1	87 ± 1**

[a]Significance was determined by testing for the probability of a least significant difference using a Student's "t" test: (*) significance at the 0.05 level, (**) significance at the 0.01 level.

Table 26. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
139	2	0 ± 0	100 ± 3	0 ± 0	100 ± 1
	4	0 ± 0	97 ± 2	0 ± 0	99 ± 1
	8	0 ± 0	95 ± 0	0 ± 0	96 ± 4
	16	0 ± 0	99 ± 1	0 ± 0	103 ± 2
140	2	20 ± 2	70 ± 6	26 ± 8	76 ± 12
	4	0 ± 0	91 ± 11	16 ± 2*	109 ± 9
	8	14 ± 1	90 ± 10	14 ± 2	105 ± 11
	16	20 ± 4	103 ± 3	11 ± 6	84 ± 6
141	2	14 ± 4	101 ± 15	21 ± 3	85 ± 10
	4	27 ± 0	77 ± 3	17 ± 4*	88 ± 2
	8	24 ± 3	89 ± 9	14 ± 3*	117 ± 5*
	16	16 ± 3	86 ± 2	20 ± 2	82 ± 2
142	2	5 ± 3	67 ± 5	12 ± 1	72 ± 2
	4	13 ± 7	76 ± 4	15 ± 0	80 ± 0
	8	7 ± 5	87 ± 9	8 ± 3	78 ± 0
	16	13 ± 0	72 ± 4	9 ± 0	85 ± 3
143	2	6 ± 1	64 ± 2	0 ± 0	72 ± 4*
	4	15 ± 7	74 ± 1	17 ± 0	74 ± 4
	8	21 ± 5	70 ± 1	30 ± 0	74 ± 4
	16	23 ± 5	91 ± 2	30 ± 5	83 ± 5

Table 26. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%DBA/1	%A x SJL	%DBA/1	%A x SJL
B. DBA/1 ⟷ (A x SJL)F_1					
94	2	0 ± 0	90 ± 3	0 ± 0	88 ± 8
	4	0 ± 0	101 ± 1	0 ± 0	103 ± 2
	8	0 ± 0	100 ± 0	0 ± 0	105 ± 4
99	2	0 ± 0	89 ± 4	0 ± 0	95 ± 6
	4	0 ± 0	92 ± 6	0 ± 0	94 ± 1
	8	0 ± 0	100 ± 1	0 ± 0	86 ± 5
100	2	4 ± 4	85 ± 7	0 ± 0	94 ± 0
	4	0 ± 0	85 ± 1	0 ± 0	95 ± 1
	8	0 ± 0	94 ± 1	0 ± 0	86 ± 1
		%CBA[b]	%DBA	%CBA[b]	%DBA
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	2	31 ± 4	86 ± 1	17 ± 2	83 ± 11
	4	15 ± 5	87 ± 0	24 ± 7	76 ± 8
	8	35 ± 0	72 ± 1	29 ± 5	77 ± 9
103	2	86 ± 1	2 ± 2	93 ± 2	9 ± 1
	4	93 ± 2	19 ± 14	95 ± 5	8 ± 8
	8	100 ± 5	2 ± 2	91 ± 5	5 ± 5
104	2	73 ± 0	26 ± 11	84 ± 0	23 ± 3
	4	72 ± 7	30 ± 10	83 ± 10	27 ± 1
	8	72 ± 7	25 ± 10	73 ± 2	21 ± 10

[b] CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 26. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a] Mixed Parental Serum %CBA[b]	Mixed Parental Serum %DBA	Allophenic Mouse Serum %CBA[b]	Allophenic Mouse Serum %DBA
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
105	2	45 ± 4	44 ± 2	56 ± 2	31 ± 1**
	4	47 ± 4	45 ± 3	51 ± 7	57 ± 3**
	8	59 ± 1	40 ± 0	35 ± 2	60 ± 2**
106	2	4 ± 3	80 ± 3	0 ± 0	113 ± 0*
	4	0 ± 0	83 ± 11	1 ± 0	103 ± 6
	8	0 ± 0	90 ± 6	4 ± 4	95 ± 9
107	2	5 ± 5	87 ± 4	24 ± 5**	81 ± 7
	4	1 ± 1	87 ± 3	27 ± 4**	67 ± 9
	8	35 ± 0	72 ± 10	16 ± 1**	93 ± 0
		%CBA[b]	%C57BL/6	%CBA[b]	%C57BL/7
D. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6					
119	2	37 ± 1	56 ± 4	38 ± 8	56 ± 4
	4	48 ± 4	53 ± 5	36 ± 9	64 ± 0
	8	44 ± 0	49 ± 2	40 ± 8	60 ± 6
	16	38 ± 1	56 ± 12	36 ± 3	58 ± 6
120	4	40 ± 1	58 ± 8	55 ± 1**	40 ± 2
	8	39 ± 1	48 ± 1	55 ± 1**	44 ± 6
125	2	13 ± 2	69 ± 5	7 ± 3	63 ± 8
	4	42 ± 5	72 ± 1	25 ± 1*	53 ± 3*
	8	21 ± 3	75 ± 5	20 ± 3	69 ± 5
127	4	34 ± 0	69 ± 3	31 ± 2	85 ± 2*
	8	26 ± 0	66 ± 5	38 ± 21	61 ± 4

Table 26. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
E. C57BL/6 ⟷ DBA/1					
101	2	--	93 ± 3	--	93 ± 10
	4	--	80 ± 7	--	88 ± 5
123	2	--	16 ± 13	--	6 ± 3
	4	--	30 ± 2	--	15 ± 13
	8	--	18 ± 9	--	7 ± 6
124	2	--	26 ± 10	--	17 ± 5
	4	--	14 ± 2	--	18 ± 0
	8	--	17 ± 2	--	20 ± 3

Table 27. Summary of the analysis of serum blocking factors using allophenic mouse sera and allophenic mouse spleen white blood cells[a]

Mouse Number	Sex	Spleen Composition %C57BL/6	Spleen Composition %A x SJL	Thymus Composition %C57BL/6	Thymus Composition %A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	♂	2	112	--	99
134	♀	2	106	--	92
135	♀	2	106	--	97
137	♀	5	89	--	105
138	♀	3	101	--	94
139	♂	1	96	--	93
140	♀	3	93	--	105
141	♀	11	102	--	100
142	♂	14	86	--	71
143	♂	23	61	--	64

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Significance was determined by testing for the probability of a least significant difference using a Student's "t" test. (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[c]Chimeric drift refers to time-dependent changes in either peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) $<$20% change, (++) $\geq$20% but $<$40% change, (+++) $\geq$40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[d]Spleen-thymus discordance refers to the difference between the relative proportions of the parental white blood cells in the spleen and thymus. (+) $<$20% discordance, (++) $\geq$20% but $<$40% discordance, (+++) $\geq$40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

Chimeric Drift[c]		Spleen-Thymus[d] Discordance	Blocking Treatement[b]			
			Mixed Parental Serum		Allophenic Mouse Serum	
Hemoglobin	PWBC		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
+	+	+	0 ± 0	90 ± 4	1 ± 1	85 ± 6
+	+	+	9 ± 3	94 ± 7	4 ± 2	101 ± 7
+	+	+	0 ± 0	96 ± 3	1 ± 3	97 ± 1
+++	+	+	1 ± 1	80 ± 5	0 ± 0	86 ± 4
+	+	+	0 ± 0	97 ± 5	0 ± 1	94 ± 4
++	+	+	0 ± 0	98 ± 2	0 ± 0	99 ± 3
++	+	+	14 ± 8	88 ± 12	17 ± 5	94 ± 11
+	-	+	20 ± 5	88 ± 9	18 ± 7	93 ± 14
++	+	+	9 ± 4	76 ± 7	11 ± 3	79 ± 5
+++	+	+	16 ± 7	75 ± 10	20 ± 11	77 ± 5

Table 27. (continued)

Mouse Number	Sex	Spleen Composition %DBA/1	Spleen Composition %A x SJL	Thymus Composition %DBA/1	Thymus Composition %A x SJL
B. DBA/1 ⟷ (A x SJL)F_1					
94	♂	7	--	--	--
99	♂	14	--	--	--
100	♂	16	--	--	--
		%CBA[e]	%DBA/1	%CBA[e]	%DBA/1
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	♀	33	74	--	--
103	♂	89	4	--	--
104	♂	70	22	--	--
105	♂	54	48	--	--
106	♀	2	102	--	--
107	♂	16	85	--	--
		%CBA[e]	%C57BL/6	%CBA[e]	%C57BL/6
D. (CBA x CBA/H-T6)F_1 ⟷ C57BL/6					
119	♀	18	58	--	--
120	♀	83	16	--	--
125	♂	17	86	25	--
127	♂	17	69	17	--
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
E. C57BL/6 ⟷ DBA/1					
101	♂	14	100	--	--
123	♂	69	7	--	85
124	♂	42	35	--	71

[e]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Chimeric Drift[c]		Spleen-Thymus[d]	Blocking Treatment[b]			
			Mixed Parental Serum		Allophenic Mouse Serum	
Hemoglobin	PWBC	Discordance	%DBA/1	%A x SJL	%DBA/1	%A x SJL
NT	NT	NT	0	97 ± 5	0	98 ± 8
NT	NT	NT	0	93 ± 5	0	91 ± 1
NT	NT	NT	1 ± 1	88 ± 4	0	92 ± 4
			%CBA[e]	%DBA/1	%CBA[e]	%DBA/1
NT	NT	NT	27 ± 9	82 ± 7	23 ± 5	78 ± 3
NT	NT	NT	93 ± 6	8 ± 8	93 ± 2	7 ± 2
NT	NT	NT	72 ± 1	27 ± 3	80 ± 5	24 ± 5
NT	NT	NT	47 ± 9	43 ± 3	53 ± 6	49 ± 13
NT	NT	NT	1 ± 3	84 ± 4	2 ± 2	103 ± 7
NT	NT	NT	14 ± 15	82 ± 7	22 ± 5	80 ± 11
			%CBA[e]	%C57BL/6	%CBA[e]	%C57BL/6
+++	+	NT	42 ± 4	53 ± 2	37 ± 2	59 ± 3
+	+	NT	40 ± 1	53 ± 5	55 ± 0**	42 ± 2
+	+	NT	25 ± 5	72 ± 3	17 ± 8	62 ± 7
++	++	NT	30 ± 4	67 ± 1	34 ± 3	73 ± 8
			%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
+	+	NT	--	87 ± 7	--	90 ± 2
+	+++	+++	--	21 ± 6	--	9 ± 4
+	++	++	--	20 ± 5	--	18 ± 1

exclusion cytotoxicity assay. Allophenic spleen white blood cells were used as the source of target cells. The mice included 10 C57BL/6 ⟷ (A x SJL)F_1 mice, 3 DBA/1 ⟷ (A x SJL)F_1 mice, 6 (CBA x CBA/H-T6)F_1 ⟷ DBA/1 mice, 4 (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 mice, and 3 C57BL/6 ⟷ DBA/1 mice. Of the 26 mice examined, 4 mice showed evidence of chimeric drift in either their peripheral white blood cell or hemoglobin compositions using a $\pm$ 40% criterion, while 22 mice were classified as being stable chimeras. Only 2 mice showed evidence of spleen-thymus discordance. Chimeric drift and spleen-thymus discordance analyses were not done on 14 mice. Mouse serum samples were examined for blocking activity using from 3-5 different serum dilutions with a constant number of target cells (1-6 x 10^6 cells/ml). All the values in Table 26 are the average of duplicate determinations at each serum dilution tested. Table 27 summarizes the data from Table 26 with each value representing the geometric mean, calculated across serum dilutions. Significant differences between the blocking activity of mixed parental serum and allophenic mouse serum are indicated in both Tables 26 and 27.

2. Allophenic mouse sera and allophenic mouse thymocytes

The analyses of the serum blocking activity of allophenic mouse serum samples on antibody mediated cytotoxicity of allophenic mouse thymocytes (1-6 x 10^6 cells/ml) are shown in Tables 28 and 29. A total of 19 mice of the same combinations as in the previous section were analyzed for blocking activity using from 2-3 different serum dilutions. All the values

Table 28. Analysis of serum blocking factors using allophenic mouse sera and allophenic mouse thymocytes

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	2	17 ± 0	100 ± 4	10 ± 3	88 ± 7
	4	15 ± 0	99 ± 4	6 ± 3	87 ± 5
	8	20 ± 4	98 ± 1	5 ± 1	92 ± 3
134	4	0 ± 0	77 ± 1	6 ± 6	89 ± 2
	8	0 ± 0	88 ± 1	13 ± 5	93 ± 1
135	2	12 ± 4	63 ± 7	8 ± 5	86 ± 2 *
	4	11 ± 0	82 ± 12	1 ± 0	96 ± 0
	8	21 ± 10	86 ± 6	0 ± 0	90 ± 0
137	2	4 ± 4	92 ± 1	23 ± 3**	93 ± 1
	4	20 ± 0	90 ± 0	29 ± 5	94 ± 0*
	8	18 ± 0	98 ± 2	34 ± 3*	91 ± 1**
138	2	29 ± 0	71 ± 7	19 ± 10	74 ± 1
	4	5 ± 4	87 ± 2	32 ± 2*	91 ± 3
139	2	10 ± 1	86 ± 1	7 ± 8	87 ± 5
	4	9 ± 6	86 ± 2	0 ± 0	88 ± 1
	8	19 ± 2	82 ± 4	0 ± 0*	80 ± 4
140	4	0 ± 0	94 ± 2	1 ± 1	95 ± 1
	8	0 ± 0	93 ± 1	14 ± 10	94 ± 1
142	4	1 ± 0	92 ± 3	0 ± 0*	91 ± 2
	8	0 ± 0	93 ± 1	0 ± 0	97 ± 2

[a]Significance was determined by testing for the probability of a least significant difference using a Student's "t" test: (*) significance at the 0.05 level, (**) significance at the 0.01 level.

Table 28. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a] Mixed Parental Serum %DBA/1	Mixed Parental Serum %A x SJL	Allophenic Mouse Serum %DBA/1	Allophenic Mouse Serum %A x SJL
B. DBA ⟷ (A x SJL)F_1					
94	4	49 ± 1	47 ± 2	57 ± 2*	53 ± 7
	8	46 ± 2	49 ± 2	49 ± 2	46 ± 3
99	4	11 ± 1	90 ± 1	19 ± 1	68 ± 8*
	8	13 ± 2	84 ± 6	23 ± 10	79 ± 5
100	4	22 ± 13	69 ± 4	30 ± 1	50 ± 6*
	8	41 ± 0	55 ± 5	32 ± 10	52 ± 3
		%CBA[b]	%DBA/1	%CBA[b]	%DBA/1
C. (CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	4	--	53 ± 1	--	58 ± 3
	8	--	76 ± 5	--	65 ± 4
103	4	--	15 ± 14	--	29 ± 0
	8	--	10 ± 10	--	27 ± 3
105	4	--	37 ± 1	--	37 ± 10
	8	--	44 ± 8	--	49 ± 2
106	4	--	97 ± 10	--	92 ± 7
	8	--	85 ± 1	--	86 ± 4
107	4	--	93 ± 0	--	100 ± 1
	8	--	97 ± 3	--	93 ± 3

[b]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 28. (continued)

Mouse Number	Reciprocal Dilution	Blocking Treatment[a]			
		Mixed Parental Serum		Allophenic Mouse Serum	
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
D. C57BL/6 ⟷ DBA/1					
101	4	--	106 ± 1	--	104 ± 1
	8	--	104 ± 4	--	104 ± 12
123	4	--	73 ± 4	--	95 ± 1
	8	--	81 ± 5	--	71 ± 9
124	4	--	76 ± 2	--	81 ± 2
	8	--	85 ± 10	--	79 ± 10

Table 29. Summary of the analysis of serum blocking factors using allophenic mouse sera and allophenic mouse thymocytes[a]

Mouse Number	Sex	Spleen Composition %C57BL/6	Spleen Composition %A x SJL	Thymus Composition %C57BL/6	Thymus Composition %A x SJL
A. C57BL/6 ⟷ (A x SJL)F_1					
133	♂	2	112	--	99
134	♀	2	106	--	92
135	♀	2	106	--	97
137	♀	5	89	--	105
138	♀	3	101	--	94
139	♂	1	96	--	93
140	♀	3	93	--	105
142	♂	14	86	--	71

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Significance was determined by testing for the probability of a least significant difference using a Student's "t" test. (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[c]Chimeric drift refers to time dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) $<$20% change, (++) $\geq$20% but $<$40% change, (+++) $\geq$40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[d]Spleen-thymus discordance refers to the difference between the relative proportions of the parental white blood cells in the spleen and thymus. (+) $<$20% discordance, (++) $\geq$20% but $<$40% discordance, (+++) $\geq$ 40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

Chimeric Drift[c]		Spleen-Thymus[d] Discordance	Blocking Treatment[b]			
			Mixed Parental Serum		Allophenic Mouse Serum	
Hemoglobin	PWBC		%C57BL/6	%A x SJL	%C57BL/6	%A x SJL
+	+	+	17 ± 2	98 ± 1	7 ± 2	89 ± 3
+	+	+	0 ± 0	82 ± 6	9 ± 4	91 ± 2
+	+	+	15 ± 4	77 ± 10	3 ± 1	91 ± 5
+++	+	+	14 ± 7	93 ± 4	29 ± 5*	93 ± 1
+	+	+	17 ± 12	79 ± 8	25 ± 7	82 ± 8
++	+	+	13 ± 5	85 ± 1	3 ± 4	85 ± 4
++	+	+	0 ± 0	93 ± 1	7 ± 7	94 ± 1
++	+	+	1 ± 1	93 ± 1	0 ± 0	94 ± 2

Table 29. (continued)

Mouse Number	Sex	Spleen Composition %DBA/1	Spleen Composition %A x SJL	Thymus Composition %DBA/1	Thymus Composition %A x SJL
B. DBA/1 ⟷ (A x SJL)F_1					
94	♂	7	--	--	--
99	♂	14	--	--	--
100	♂	16	--	--	--
		%CBA[e]	%DBA/1	%CBA[e]	%DBA/1
C. CBA x CBA/H-T6)F_1 ⟷ DBA/1					
97	♀	33	74	--	--
103	♂	89	4	--	--
105	♂	54	48	--	--
106	♀	2	102	--	--
107	♂	16	85	--	--
		%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
D. C57BL/6 ⟷ DBA/1					
101	♂	14	100	--	--
123	♂	69	7	--	85
124	♂	42	35	--	71

[e]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Chimeric Drift[c]		Spleen-Thymus[d] Discordance	Blocking Treatment[b]			
			Mixed Parental Serum		Allophenic Mouse Serum	
Hemoglobin	PWBC		%DBA/1	%A x SJL	%DBA/1	%A x SJL
NT	NT	NT	47 ± 1	48 ± 1	53 ± 5	50 ± 4
NT	NT	NT	12 ± 1	87 ± 3	21 ± 6	73 ± 2
NT	NT	NT	31 ± 10	62 ± 7	31 ± 1	51 ± 1
			%CBA[e]	%DBA/1	%CBA[e]	%DBA/1
NT	NT	NT	--	65 ± 10	--	62 ± 3
NT	NT	NT	--	13 ± 3	--	28 ± 1
NT	NT	NT	--	41 ± 1	--	43 ± 7
NT	NT	NT	--	91 ± 6	--	89 ± 3
NT	NT	NT	--	95 ± 2	--	97 ± 4
			%C57BL/6	%DBA/1	%C57BL/6	%DBA/1
+	+	NT	--	105 ± 1	--	104 ± 1
+	+++	+++	--	77 ± 4	--	83 ± 2
+	++	+++	--	81 ± 5	--	80 ± 1

in Table 28 are the average of duplicate determinations at each serum dilution tested. Table 29 summarizes the data from Table 28 with each value representing the geometric mean calculated across serum dilutions. Significant differences between the blocking activity of mixed parental serum and allophenic mouse serum are indicated in both Tables 28 and 29.

3. Allophenic mouse sera and parental spleen white blood cells

The analyses of serum blocking activity of allophenic mouse serum samples on antibody mediated cytotoxicity of parental spleen white blood cells for 45 mice of combinations-- 16 C57BL/6 ⟷ (A x SJL)F_1, 13 (CBA x CBA/H-T6)F_1 ⟷ DBA/1, 1 (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, 3 C57BL/6 ⟷ DBA/1, and 12 C57BL/6 ⟷ A mice--are shown in Tables 31, 32, 33, and 34, respectively. An initial serum dilution of 1:5 was used for all experiments. All the values in Tables 30, 31, 32, 33, and 34 are the average of duplicate determinations.

Table 30. Analysis of serum blocking activity using C57BL/6 ⟷ (A x SJL)F_1 mouse sera and parental spleen white blood cells[a]

Mouse Number	Sex	Spleen Composition %C57BL/6	Spleen Composition %A x SJL	Thymus Composition %C57BL/6	Thymus Composition %A x SJL
133	♂	2	112	--	99
134	♀	2	106	--	92
135	♀	2	106	--	97
137	♀	5	89	--	105
138	♀	3	101	--	94
139	♂	1	96	--	93
140	♀	3	93	--	105
141	♀	11	102	--	100
142	♂	14	86	--	71
143	♂	23	61	--	64
148	♀	2	102	5	90
149	♂	4	102	7	92
150	♀	8	102	0	97
151	♂	4	103	0	99
152	♀	6	107	0	95
153	♀	9	95	49	46

[a]Spleen and thymus composition of allophenic mice are based are duplicate values determined at the time of sacrifice. Data were taken from Table 22.

[b]Control samples preincubated with a 1:5 dilution of either type of parental serum or an equal ratio of mixed parental serum gave average cytotoxicity values of 53 ± 10% and 51 ± 8% for C57BL/6 and (A x SJL)F_1 target cells respectively.

[c]Significance was determined by testing for least significant differences between normal mouse serum controls and allophenic mouse serum samples at a 50% cytotoxic dose of antiserum using Cochran's approximate t-test (Snedecor and Cochran, 1967). (*) significant at the 0.05 level, (**) significant at the 0.01 level.

[d]Chimeric drift refers to time-dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) ≤20% change, (++) ≥20% change but <40% change, (+++) ≥40% change, (-) no change, and (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[e]Spleen-thymus discordance refers to the difference betwen relative proportions of the parental blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

Chimeric Drift[d]		Spleen-Thymus[e] Discordance	Blocking Treatment[b,c] Allophenic Mouse Sera	
Hemoglobin	PWBC		%C57BL/6	%A x SJL
+	+	+	73 ± 10*	42 ± 12
+	+	+	68 ± 1*	42 ± 8
+	+	+	43 ± 2	51 ± 17
+++	+	+	47 ± 5	62 ± 10*
+	+	+	64 ± 1	51 ± 7
++	+	+	67 ± 2	62 ± 0*
++	+	+	49 ± 1	57 ± 2
+	-	+	47 ± 6	57 ± 3
++	+	+	47 ± 2	61 ± 5*
+++	+	+	46 ± 9	56 ± 3
NT	+	+	57 ± 4	49 ± 3
NT	+	+	57 ± 2	49 ± 2
NT	+	+	58 ± 2	59 ± 2
NT	+	+	60 ± 6	53 ± 2
NT	+	+	53 ± 4	51 ± 0
NT	+	+++	52 ± 1	53 ± 1

Table 31. Analysis of serum blocking activity using (CBA x CBA/H-T6)F_1 ⟷ DBA/1 mouse sera and parental spleen white blood cells[a1]

Mouse Number	Sex	Spleen Composition		Thymus Composition	
		%CBA[f]	%DBA/1	%CBA[f]	%DBA/1
97	♀	33	74	--	--
103	♂	89	4	--	--
104	♂	70	22	--	--
105	♂	54	48	--	--
107	♂	16	85	--	--
144	♂	--	60	--	95
146	♂	100	2	--	3
154	♂	104	7	--	8
163	♀	7	104	--	84
164	♀	16	93	--	109
166	♂	81	40	--	0
174	♂	101	7		0
175	♀	83	24		7

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Control samples preincubated with a 1:5 dilution of either type of parental serum or an equal ratio of mixed parental serum gave mean cytotoxicity values of 53 ± 10% and 49 ± 10% for (CBA x CBA/H-T6)F_1 and DBA/1 target cells, respectively.

[c]Significance was determined by testing for least significant differences between normal mouse serum controls and allophenic mouse serum samples at a 50% cytotoxic dose of antiserum, using Cochran's approximate t-test (Snedecor and Cochran, 1967). (*) significant at the 0.05 level. (**) significance at the 0.01 level.

[d]Chimeric drift refers to time-dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) <20% change, (++) ≤ 20% but <40% change, (+++) ≥40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[e]Spleen-thymus dicordance refers to the difference between the relative proportions of the parental white blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

[f]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Chimeric Drift[d]		Spleen-Thymus[e] Discordance	Blocking Treatment[b,c] Allophenic Mouse Sera	
Hemoglobin	PWBC		%CBA[f]	%DBA/1
NT	NT	NT	60 ± 8	43 ± 2
NT	NT	NT	52 ± 4	70 ± 0**
NT	NT	NT	50 ± 17	43 ± 5
NT	NT	NT	49 ± 5	44 ± 7
NT	NT	NT	74 ± 9	46 ± 1
NT	++	++	46 ± 2	64 ± 2*
NT	-	+	56 ± 1	54 ± 8
NT	+	+	54 ± 8	51 ± 3
NT	+	+	52 ± 10	49 ± 6
NT	+	+	65 ± 2	70 ± 2**
NT	+	+++	60 ± 3	49 ± 2
NT	NT	+	51 ± 3	52 ± 0
NT	NT	+	50 ± 21	55 ± 6

Table 32. Analysis of serum blocking activity using (CBA x CBA/H-T6)F_1 ⟷ C57BL/6 mouse sera and parental spleen white blood cells[a]

Mouse Number	Sex	Spleen Composition %CBA[f]	Spleen Composition %C57BL/6	Thymus Composition %CBA[f]	Thymus Composition %C57BL/6	Chimeric Drift[d] Hemoglobin	Chimeric Drift[d] PWBC	Spleen-Thymus[e] Discordance	Blocking Treatment[b,c] Allophenic Mouse Sera %CBA[f]	Blocking Treatment[b,c] Allophenic Mouse Sera %C57BL/6
127	♂	32	69	17	-	++	++	+	41 ± 11	60 ± 1

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Control samples preincubated with a 1:5 dilution of either type of parental serum or an equal ratio of mixed parental serum gave mean cytotoxicity values of 71 ± 11% and 67 ± 10% for (CBA x CBA/H-T6)F_1 and C57BL/6 target cells, respectively.

[c]Significance was determined by testing for least significant differences between normal mouse serum controls and allophenic mouse serum samples at a 50% cytotoxic dose of antiserum, using Cochrans approximate t-test (Snedecor and Cochran, 1967). (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[d]Chimeric drift refers to time-dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) <20% change, (++) ≥20% but <40% change, (+++) ≥40%, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[e]Spleen-thymus discordance refers to the difference between the relative proportions of parental white blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

[f]CBA is an abbreviation for (CBA x CBA/H-T6)F_1.

Table 33. Analysis of serum blocking activity using C57BL/6 ⟷ DBA/1 mouse sera and parental spleen white blood cells[a]

Mouse Number	Sex	Spleen Composition %C57BL/6	Spleen Composition %DBA/1	Thymus Composition %C57BL/6	Thymus Composition %DBA/1	Chimeric Drift[d] Hemoglobin	Chimeric Drift[d] PWBC	Spleen-Thymus[e] Discordance	Blocking Treatment[b,c] Allophenic Mouse Sera %C57BL/6	Blocking Treatment[b,c] Allophenic Mouse Sera %DBA/1
101	♂	14	100	--	--	+	-	NT	69 ± 4	57 ± 9
123	♂	69	7	--	85	+	+++	+++	69 ± 4	51 ± 4
124	♂	42	35	--	71	+	++	++	58 ± 8	41 ± 8

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Control samples preincubated with 1:5 dilution of either type of parental serum or an equal ratio of mixed parental serum gave mean cytotoxicity values of 71 ± 10% and 52 ± 12% for C57BL/6 and DBA/1 target cells, respectively.

[c]Significance was determined by testing for least significant differences between normal mouse serum controls and allophenic mouse serum samples at a 50% cytotoxic dose of antiserum, using Cochran's approximate t-test (Snedecor and Cochran, 1967). (*) significance at the 0.05 level, (**) significance at the 0.01 level.

[d]Chimeric drift refers to time-dependent changes in either the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) <20% change, (++) ≥20% but <40% change, (+++) ≥40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[e]Spleen-thymus discordance refers to the difference between relative proportions of the parental white blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

Table 34. Analysis of serum blocking activity using C57BL/6 ⟷ A mouse sera and parental spleen white blood cells[a]

Mouse Number	Sex	Spleen Composition %C57BL/6	Spleen Composition %A	Thymus Composition %C57BL/6	Thymus Composition %A
157	♂	95	16	90	5
158	♀	97	20	107	0
159	♀	11	86	57	110
160	♂	72	26	111	0
162	♂	63	46	73	47
167	♂	106	13	102	8
168	♂	95	29	93	13
170	♀	1	99	2	100
171	♂	1	98	8	97
173	♂	59	53	32	73
187	♀	84	8	105	0
188	♀	--	--	--	--

[a]Spleen and thymus composition of allophenic mice are based on duplicate values determined at the time of sacrifice using the trypan blue dye exclusion assay. Data were taken from Table 22.

[b]Control samples preincubated with a 1:5 dilution of either type of parental serum or an equal ratio of mixed parental serum gave average cytotoxocity values of 55 ± 12% and 56 ± 8% for C57BL/6 and A target cells, respectively.

[c]Significance was determined by testing for least significant differences between normal nouse serum controls and allophenic mouse serum samples at a 50% cytotoxic dose of antiserum using Cochran's approximate t-test (Snedecor and Cochran, 1967). (*) significance at the 0.5 level, (**) significance at the .01 level.

[d]Chimeric drift refers to time-dependent changes in the peripheral white blood cells (PWBC) or hemoglobin (Hb). (+) <20% change, (++) ≥20% but <40% change, (+++) ≥40% change, (-) no change, (NT) not tested. Data were taken from Tables 13, 14, 15, 16, 17, 18, and 19.

[e]Spleen-thymus discordance refers to the difference between the relative proportions of the parental white blood cells in the spleen and thymus. (+) <20% discordance, (++) ≥20% but <40% discordance, (+++) ≥40% discordance, (-) no discordance, (NT) not tested. Data were taken from Tables 22 and 25.

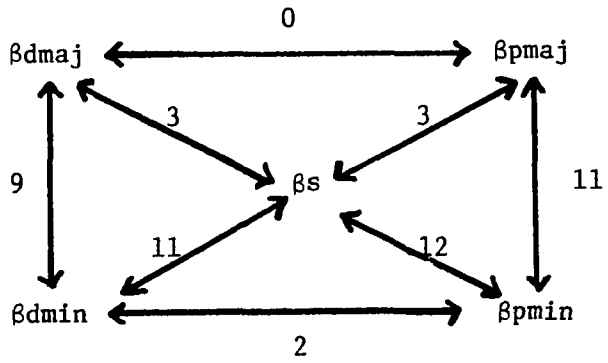
Chimeric Drift[d]		Spleen-Thymus[e] Discordance	Blocking Treatment[b,c] Allophenic Mouse Sera	
Hemoglobin	PWBC		%C57BL/6	%A
+	+	+	52 ± 1	59 ± 10
++	+	++	55 ± 4	49 ± 1
+++	+	+++	57 ± 3	48 ± 1
+	++	++	60 ± 5	44 ± 2
+	+++	+	50 ± 1	47 ± 5
+	++	+	46 ± 9	48 ± 4
+	+	+	44 ± 3	33 ± 1*
+	+	+	44 ± 3	43 ± 2
+	+	+	52 ± 5	40 ± 3
+	+++	++	46 ± 1	43 ± 3
-	+++	+	49 ± 1	51 ± 9
+	+++	NT	45 ± 4	52 ± 9

IV. DISCUSSION

A. Chimeric Drift

1. Murine hemoglobin

The adult mouse hemoglobin is a molecule (65,000 daltons, M.W.) of four globin chains (two α chains and two β chains) each of which is bound to a heme residue in noncovalent linkage (Russell and Bernstein, 1966). In 1962, Hutton and his colleagues using starch gel electrophoresis showed that some inbred strains of mice have an adult β-chain doublet producing two β-chains in unequal amounts, designated Hbb^d. Other strains of mice have an adult β-chain singlet designated Hbb^s (Hutton et al., 1962a, b). Morton (1966) reported a third allele at the β-chain locus of mice, designated Hbb^p with the p hemolysate bearing some resemblance to the d hemolysate. In 1972, Gilman isolated and sequenced the two β-chains from the d hemolysate and demonstrated that each possesses a structurally different β-chain. In the d hemolysate, $\beta^{d\,min}$ represents approximately 20% of the total, while $\beta^{d\,maj}$ accounts for the remaining 80%. He further demonstrated that Hbb^p was a variant form of the Hbb^d allele, since it produces a slightly different minor β-chain, $\beta^{p\,min}$. The difference was seen at two positions in the minor β-chains (Gilman, 1972). Figure 28 summarizes the differences in amino acid sequences between the three different types of β-chains in laboratory mice. Hemoglobins Hbb^s, Hbb^d and Hbb^p are coded for by three separate loci; two (Hbb^s, Hbb^d or Hbb^p) of the three segregate



	9	13	16	20	22	23	58	76	76	77	80	104	139
βs	Ala	Gly	Gly	Ala	Glu	Val	Ala	Asp	Asn	His	*	Met	Ala
βdmaj	Ala	Cys	Gly	Ser	Glx	Val	Ala	Asp	Asn	His	*	Met	(Thr)
βdmin	Ser	(Cys)	Ala	Pro	Glx	Val	Pro	Glu	Lys	Asn	Asn	Alx	(Thr)
βpmin	Syr	Cys	Ala	Pro	Ala	Ile	Pro	Glx	Lys	Asn	Asn	?	(Thr)

Uncommon symbols are Syr, serine or cysteine; Alx, probably alanine, possibly valine; ?, an unknown residue; *, probably serine; **, not arginine; ***, not methionine. Standard abbreviations are: A, Ala, alanine; R, Arg, arginine; N, Asn, asparagine; D, Asp, aspartic acid; C, Cys, cysteine; E, Glu, glutamic acid; Z, Glx, glutamic acid or glutamine; G, Gly, glycine; H, His, histidine; I, Ile, isoleucine; L, Leu, leucine; K, Lys, lysine; M, Met, methionine; F, Phe, phenylalanine; P, Pro, proline; S, Ser, serine; T, Thr, threonine; W, Trp, tryptophan; Y, Tyr, tyrosine; V, Val, valine. Parentheses around a residue mean that tryptic peptide composition data are all that are available, and that the position in the sequence has been inferred by homology.

Figure 28. A comparison of Mus musculus musculus β chains β^{s}, β^{dmaj}, β^{dmin}, and β^{pmin} at the positions where differences are found (Gilman, 1972)

as separate units in breeding experiments (Gilman, 1974). It has been suggested that Hbb^d or Hbb^p loci may actually contain two almost identical DNA sequences that may code for the two slightly different hemoglobin β-chains (Gilman, 1974). The breeding unit alleles coding for the hemoglobin β-chain in mice have been mapped to a segment of chromosome 7 near the albino locus (Russell and McFarland, 1974).

Some laboratory mice have also been shown to have hemoglobin α-chains that can exist in polymorphic forms (Gilman, 1974). The locus coding for genetic polymorphism seen in the α-chains has been designated, Hba (Russell and Bernstein, 1966). This locus has been mapped to a region of chromosome 11 (Russell and McFarland, 1974). Despite the polymorphic forms of Hba no electrophoretically distinct products are known to exist in laboratory mice (Gilman, 1974).

Russell and Bernstein (1966) have summarized the techniques used to separate and characterize mouse hemoglobin. Diffuse hemoglobins (Hbb^d or Hbb^p) are more soluble in buffered salt solutions, over a wider pH and molarity range than any of the single hemoglobins (Hbb^s). Associated with this difference in solubility is a difference in crystal formation. Typically, homogenous single hemoglobin precipitates from buffered salt solutions as large hexagonal crystals, while the diffuse hemoglobin appears as a flocculent amorphorous precipitate. During column chromatography over Amberlite, the single hemoglobin appears as a single major peak, while the major band of the diffuse appears as a major single peak in the same regions as the single hemoglobin, plus an additional minor component.

In order to quantitate mixtures of Hbb^s and Hbb^d we developed the technique of polyacrylamide gel isoelectric focusing in our laboratory. The technique proved to be simple, rapid, and quantitative. Complete separation of Hbb^s and Hbb^d was achieved by chemically modifying the cysteine at position 13 of the Hbb^d, leaving the Hbb^s glycine at position 13 unaltered (Wegmann and Gilman, 1970). The technique we developed now seems to be the method of choice in analyzing murine hemoglobins.

2. Analysis of changes in red blood cell and white blood cell populations in allophenic mice

This study demonstrates that allophenic mice can show marked changes in their red blood cell and white blood cell populations with time. The results of the analysis of 85 allophenic mice, shown in Tables 13, 14, 15, 16, 17, 18, and 19 are depicted in Figures 21, 22, 23, 24, 25, and 26. A summary of the results of the analysis of all mice is shown in Table 20. First of all, it is seen that at least some of the mice of parental types C57BL/6 ⟷ (A x SJL)F_1, (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, C57BL/6 ⟷ DBA/1 and C57BL/6 ⟷ A, but not C57BL/10Sn ⟷ A mice, showed significant changes in their hemoglobin compositions. Some mice of all six types showed significant changes in their peripheral white blood cells. Second, it is seen that 9 of 71 or 13% of the mice showed significant changes in their hemoglobin compositions, whereas 13 of 85 or 15% of the mice showed significant changes in their peripheral white blood cell composition. Only one mouse (84) showed a change in both hemoglobin and peripheral white blood cell composition. Thus, a total of 21 of 84 or 25% of the total

number of allophenic mice tested showed a significant change in their hemoglobin composition, their peripheral white blood cell composition, or both. These changes have been termed chimeric drift. Allophenic mice have been classified as "stable" or "unstable" depending on whether or not they show significant chimeric drift.

It should be noted that the number of mice showing significant chimeric drift as shown in Table 20, is a minimum number, since significant differences were considered to be $\pm$ 40%. Deciding what constitutes chimeric drift in this system is not a simple problem, since the error in individual determinations varies between $\pm$ 10% and $\pm$ 20%. If the error in each determination was closer to $\pm$ 10% than $\pm$ 20%, so that significant drift might be considered to be changes greater than $\pm$ 20%, then 24 of 71 or 34% of the mice showed significant changes in their hemoglobin composition, 28 of 85 or 33% showed significant changes in their peripheral white blood cell composition and 51 of 85 or 60% showed changes in both. However, for the purpose of discussion, the $\pm$ 40% criterion will be used, since the overall conclusions are the same and the significance of the drift is more certain. Using changes of $\pm$ 40% as the criterion for the definition of chimeric drift, there is no question that any observed drift is not simply because of experimental error.

From Figures 21, 22, 23, 24, and 25, and Table 19, it is seen that chimeric drift is independent of the age, sex, and coat color of a mouse. Mice may show significant drift at any age, with apparently no more likelihood of drift in young mice than in old mice. Of the 21 mice with

significant drift, 10 were phenotypic males and 11 were phenotypic females. Of the total number of mice, there were 50 males and 35 females. Among all of the mice there were 46 multicolored mice and 39 single-colored mice. This confirms a number of earlier studies (Mintz, 1969b; Mintz and Palm, 1969; Wegmann, 1973) which have suggested that the coat color and organ composition of individual allophenic mice are subjected to different selective pressures and may be independent. This is especially true of coat color compared with blood cell composition, since the former, with the possible exception of agouti versus nonagouti chimeras, is of ectodermal origin whereas the latter is of mesodermal origin (Wegmann and Gilman, 1970). The drift in peripheral white blood cell composition was found in 13 mice. Of the 13 mice, 12 showed significant decreases in the number of C57BL/6 or C57BL/10Sn peripheral white blood cells. One of the mice (212) showed a significant increase in C57BL/10Sn cells, while three mice (123, 158 and 214) showed a significant increase followed by a decrease at a later time. Thus, the shift in the peripheral white blood cell population is not unidirectional.

This is demonstrated more dramatically in Figure 26, with the tissue typing of nine C57BL/10 Sn $\longleftrightarrow$ A mice using two types of antisera. These observations confirm an early study (Warner et al., 1976) on chimeric drift of the peripheral white blood cell populations in C57BL/6 $\longleftrightarrow$ (A x SJL)F_1 allophenic mice.

The results on drift in the hemoglobin composition of the mice can be similarly analyzed. It is seen that five of the mice (84, 85, 119,

143, and 191) showed a significant decrease in the amount of C57BL/6 hemoglobin, three of the mice (76, 114, and 159) showed a significant increase in the amount of C57BL/6 hemoglobin and one mouse (137) showed a significant increase and then a significant decrease in the amount of C57BL/6 hemoglobin. Thus, there is no apparent preference for one cell type or the other, and there is no evidence for unidirectional shifts in hemoglobin composition. This result is somewhat at odds with those of Mintz and Palm (1969), who studied H-2 antigens on the surface of erythrocytes of 34 C3H(f) ⟷ C57BL/6 allophenic mice. Their data suggested that all shifts were in the direction of the C57BL/6 parental type. However, only a limited number of determinations were made, so that shifts in the opposite direction from C57BL/6 may not have been present in their sample or else could have easily escaped detection. Similarly, West (1977) reported preferential shifts in the hemoglobin of 6 out of 8 C3H/BiMcL ⟷ C57BL/McL mice in the direction of the C57BL/McL parental type. He concluded that time dependent changes in erythropoietic tissue may be the result of differential rates of mitotic activity that contributes to strain-dependent, tissue-specific selective pressures in allophenic mice. In contrast to West's findings is the present study, which has shown that chimeric drift in the five combinations of allophenic mice may occur in either parental direction and fluctuate back and forth between the two parental cell types.

Shifts in the erythrocyte composition of chimeras in other species have been documented in the past. In both cattle (Stone et al., 1964)

and marmoset (Gengozian et al., 1969) chimeras, there is a spontaneous change in the proportion of each cell population with age. It must be remembered that the cattle and marmoset chimeras are different from allophenic mice in that they are secondary chimeras, and therefore, chimeric only in their blood systems. The allophenic mice, on the other hand, are primary chimeras, and may be chimeric in any or all of their tissues. The random shifts in the erythrocyte population of allophenic mice very closely resemble the random shifts seen in cattle chimeras; both twins usually shift in the same direction, even though two sets of twins may shift in opposite directions. Thus, there is an apparent genetic influence on the direction of drift in secondary chimeras, which may or may not apply to the shifts seen in allophenic mice.

Table 21 summarizes the statistical analysis of the correlation of hemoglobin and peripheral white blood cell composition of 71 allophenic mice. It is seen that there is a good correlation of hemoglobin and peripheral white blood cell composition when all of the mice are considered in each of the five allophenic combinations. When only the stable mice are considered, this correlation becomes even better. However, when the unstable mice are considered separately, the five combinations of allophenic mice show poor or no correlation of the two parameters. Figure 27 shows the distribution of the hemoglobin composition of the 15 C57BL/6 ⟷ A mice as a function of their peripheral white blood cell composition at any give time point. As previously demonstrated in Table 21, there is a good statistical correlation of the data. Thus, there is strong support

for the concept that erythrocytes and white blood cells arise from the same pluripotent hematopoietic stem cell during embryogenesis (Till et al., 1975). However, the mechanism for the maintenance of the two cell populations in the adult may not be coordinated (Gandini et al., 1968; Mintz and Palm, 1969; Wegmann and Gilman, 1970). In another study (Berntson, 1978) in which the IgG2a allotype composition was compared to the hemoglobin composition of eight (CBA x CBA/H-T6)F_1 ←→ C57BL/6 mice, only a slight correlation of these two parameters was observed, thus further supporting the concept of separate control mechanisms of red blood cell and white blood cell populations.

The lack of concordance of drift in the hemoglobin and peripheral white blood cell population leads to some speculations about the mechanisms of chimeric drift in allophenic mice. One might wonder whether chimeric drift can be taken as evidence for the breakdown of the tolerance mechanism in the allophenic mice. If this were true, there might be a difference in the ability to detect serum-blocking factors or suppressor cells in unstable versus stable allophenic mice. However, it has been shown that blocking factors were not detected in allophenic mouse sera regardless of whether the source of serum was from a stable or unstable mouse (see Discussion, Section C).

Finally, it seems apparent from these data that there is no simple cause and effect mechanism to account for the differential drift in the peripheral white blood cell and erythrocyte populations. For instance, if the amount of C57BL/6 or C57BL/10Sn hemoglobin had always gone down

as the number of C57BL/6 or C57BL/10Sn white blood cells went up, a cause and effect relationship might be postulated. This is obviously not the case among the unstable mice since some of them show a coordinate decrease in both C57BL/6 hemoglobin and peripheral white blood cells, whereas others show either one or the other parameter rising while the other is falling. No periodicity of these markers could be detected by analyzing mice at a weekly interval for six weeks (Figures 25 and 26). However, it is possible that a undetected periodicity of the data might have been detected by analyzing the mice weekly for 6-12 months. On the other hand, it is strongly suggested that drift in the two cell populations in adult mice is independently determined by the environment and the mechanisms involved in drift are not the same as those responsible for the maintenance of tolerance.

B. Composition of the Immune System of Allophenic Mice

This study describes the composiiton of the immune system of eight different types of allophenic mice, based on the presence of different surface antigens on the two types of parental cells, as detected by antibody mediated cytotoxicity. As is seen in Table 22, the 93 mice studied covered the entire range of possible chimeric mixtures. This probably reflects the chance differential death of one parental cell type or other during early embryogenesis (Mullen and Whitten, 1971). It is seen that chimerism for coat color does not necessarily predict the internal composition of any given mouse; this is in agreement with the earlier

observations of Mintz and Palm (1969). In fact, the internal composition of the mice seems to be independent of the coat color, sex or age of the mice at the time of sacrifice.

Table 23 demonstrates the additivity of the different parental cell types in the eight combinations of allophenic mice. In no case do the figures add up to significantly more than 100%. This may be evidence for a small percentage of cells that might be hybrids. The possibility of transfer of genetic information or spontaneous fusion of a small number of parental cells in allophenic mice has never been vigorously excluded (Munro et al., 1974a). One previous study suggests that there is no fusion of cells in allophenic mice (Bona et al., 1974). However, it is clear from these data that the vast majority of cells in these chimeric animals have one or the other parental type of cell surface antigens. In no case do the values add up to significantly less than 100%. This can be interpreted to mean that allophenic mice do not have a specific factor in their spleens which prevents complete killing with the antisera. This is consistent with the observations of the absence of serum blocking factors previously reported in allophenic mice (Meo et al., 1973; Festenstein et al., 1975; von Boehmer et al., 1975a)(see Discussion, Section C).

Table 24 summarizes the correlation of the peripheral blood, spleen and thymus cells of the eight different combinations of mice. It is seen in Table 24 that there is an excellent correlation of the peripheral blood composition with the spleen composition of all mice of a parental combination. Since blood contains 70% T cells while spleen contains 35% T cells

(Gornish et al., 1972), one might speculate that the relative proportion of the two parental types of T cells is the same in the blood and spleen. Furthermore, the correlation of the thymus composition with the peripheral blood cell or the spleen compositions is not statistically significant in most allophenic mice and does not correlate well as the peripheral blood and spleen composition, with the exception of the (CBA x CBA/H-T6)$F_1 \longleftrightarrow$ DBA/1 mice and C57BL/6$\longleftrightarrow$A mice. These two combinations of mice have an excellent correlation between these markers. The explanation for this discrepancy is not clear. It is possible that all of the lymphomyeloid cells have a common origin during embryogenesis and then diverge due to differential proliferation of one cell type or the other in the adult allophenic animal. Thus, it would be likely that the particular strains of mice used in the allophenic combinations would determine the degree of concordance of the various cell populations in the adult mice. This conclusion would explain the data reported and those of Gornish (Gornish et al., 1972) and Ford (Ford et al., 1975) (see Introduction).

Figures 29, 30 and 31, respectively, show the distribution of the parental strain peripheral white blood cells, spleen white blood cells, and thymocytes in allophenic mice. As can be seen, the parental lymphoid cells approximate an uniform distribution with the ratio of a parental type occurring at an almost equal frequency, with the exceptions the two ends. Similar results are also seen with respect to coat color. These distributions are consistent with and predicted from the work of Falconer and Avery (1968) who similarly observed an approximately flat distribution

Figure 29. Distribution of parental strain 1 peripheral white blood cells (top) and coat color (bottom) in 92 allophenic mice of parental strain combinations (33) C57BL/6 ⟷ (A x SJL)F_1, (3) DBA/1 ⟷ (A x SJL)F_1, (3) DBA/1 ⟷ A, (16) (CBA x CBA/H-T6)F_1 ⟷ DBA/1, (16) (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, (5) (CBA x CBA/H-T6)F_1 ⟷ A, (3) C57BL/6 ⟷ DBA/1, and (13) C57BL/6 ⟷ A mice. Peripheral white blood cell (PWBC) composition was determined at the time of sacrifice using the trypan blue dye exclusion assay. Coat color phenotype was estimated visually at weaning. Data were taken from Table 22.

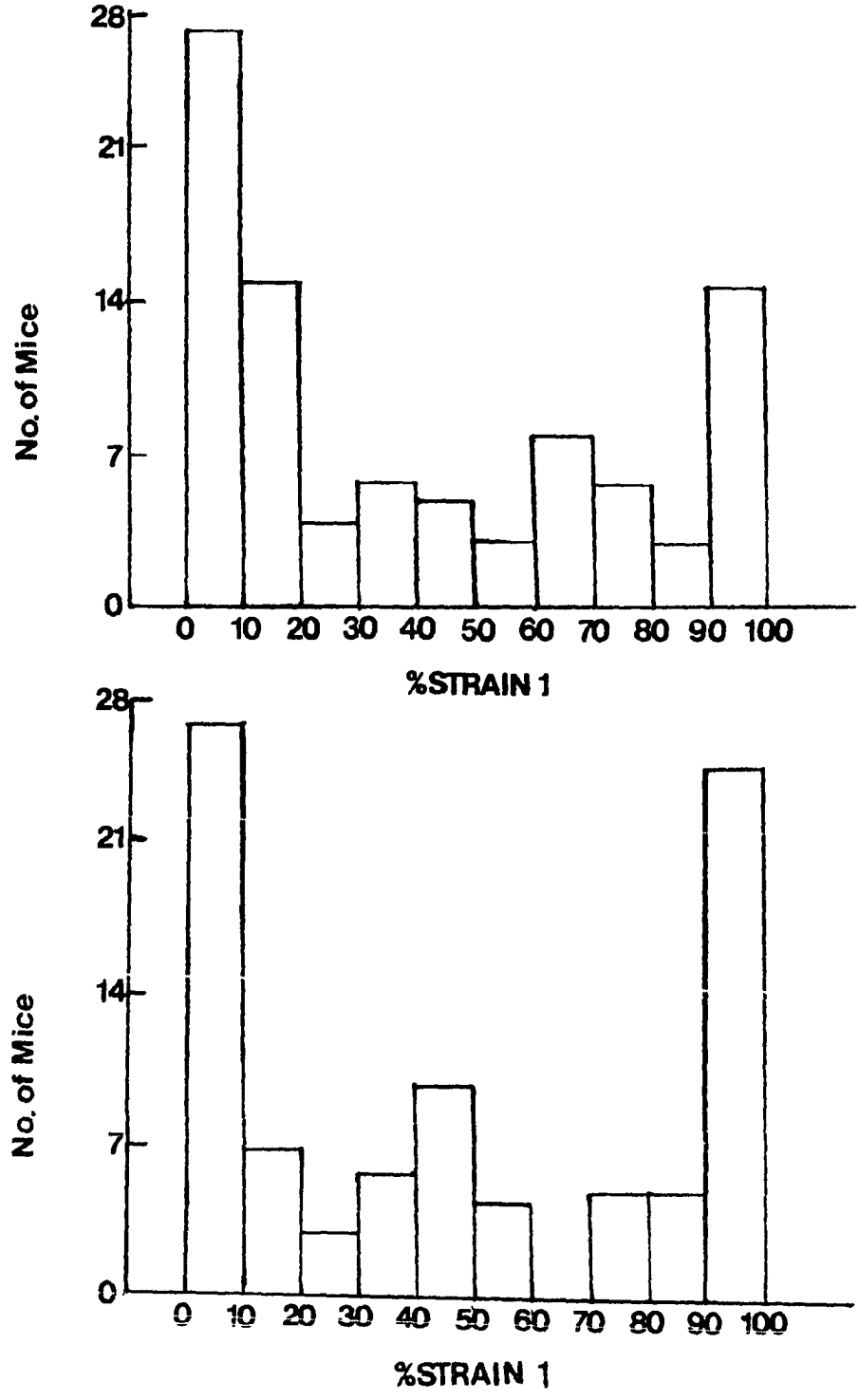
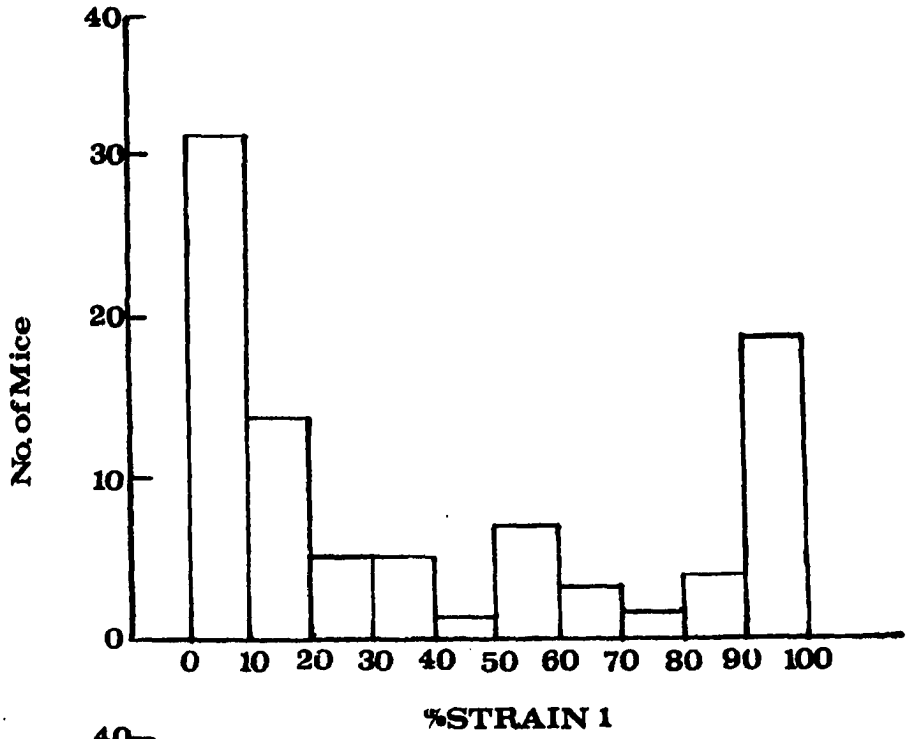

28
21
14
7
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1
28
21
14
7
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1

Figure 30. Distribution of parental strain 1 spleen white blood cells (top) and coat color (bottom) in 92 allophenic mice of parental strain combinations (33) C57BL/6 ⟷ (A x SJL)F_1, (3) DBA/1 ⟷ (A x SJL)F_1, (3) DBA/1 ⟷ A, (16) (CBA x CBA/H-T6)F_1 ⟷ DBA/1, (16) (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, (5) (CBA x CBA/H-T6)F_1 ⟷ A, (3) C57BL/6 ⟷ DBA/1, and (13) C57BL/6 ⟷ A mice. [Spleen white blood cell composition was determined at the time of sacrifice using the trypan blue dye exclusion assay. Coat color phenotype was estimated visually at weaning. Data were taken from Table 22.]


40
30
20
10
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1

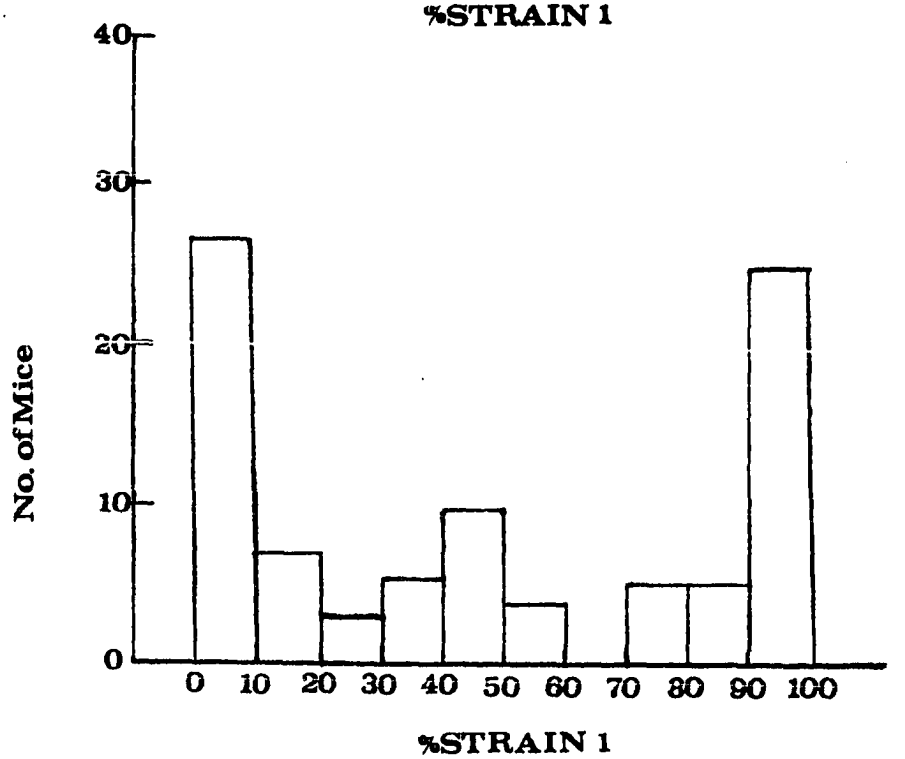
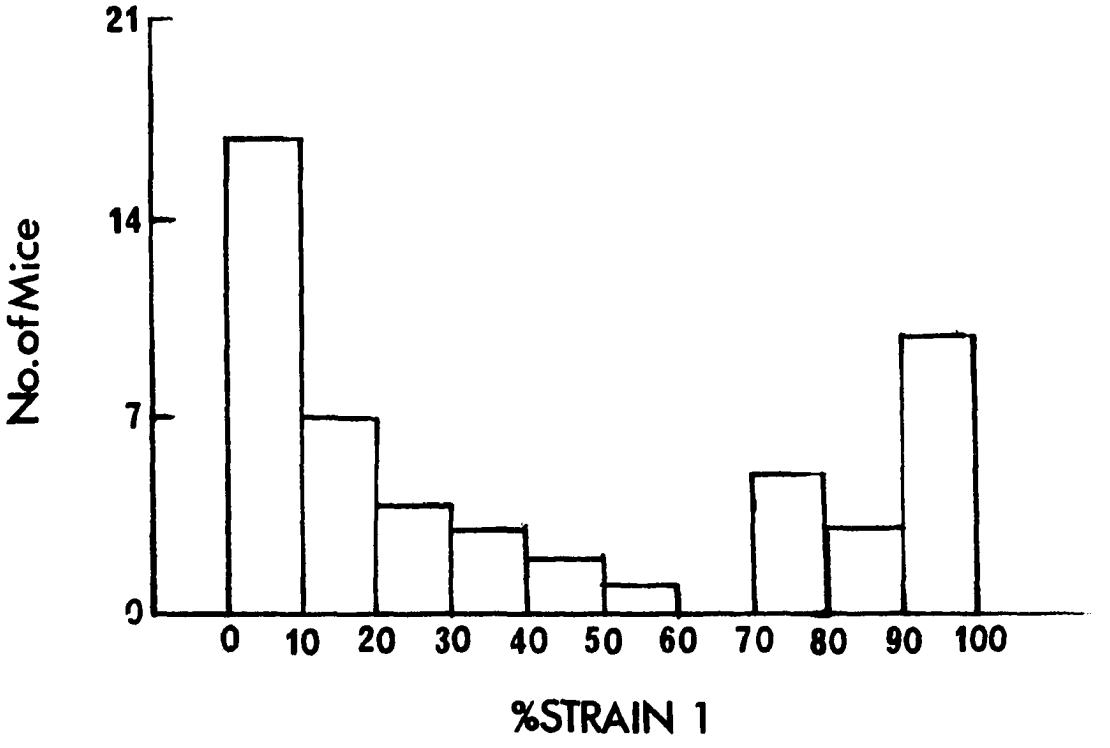
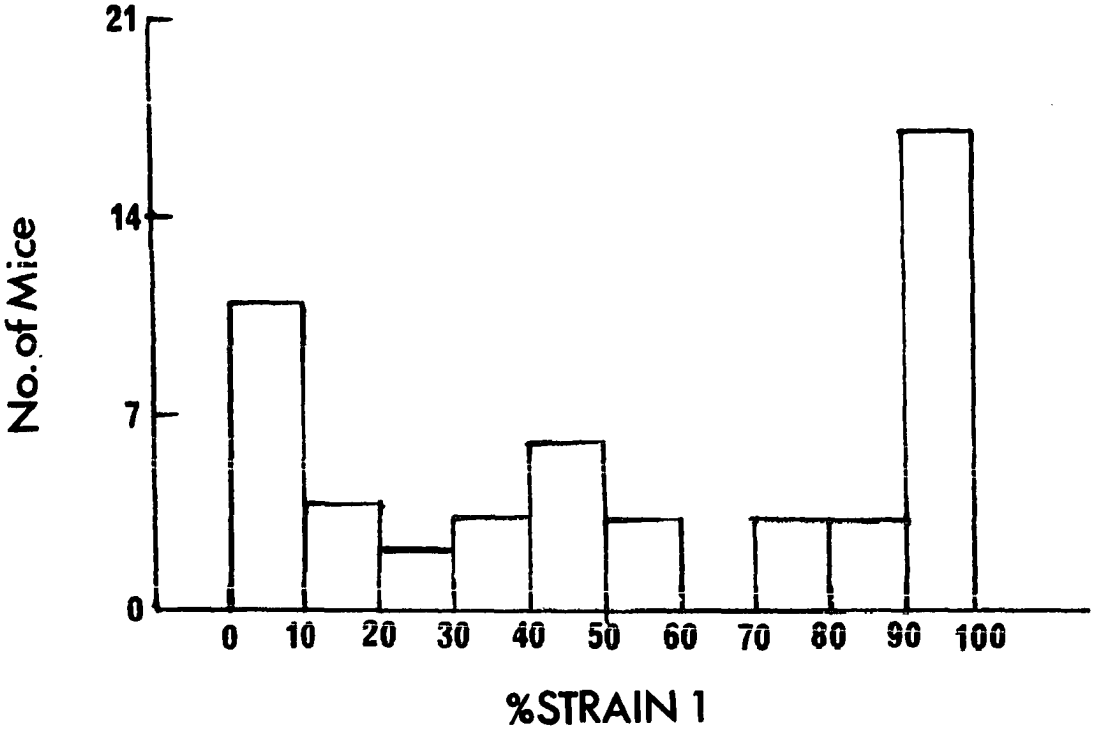

40
30
20
10
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1

Figure 31. Distribution of parental strain 1 thymocytes (top) and coat color (bottom) in 52 allophenic mice of parental strain combinations (16) C57BL/6 ⟷ (A x SJL)F_1, (3) DBA/1 ⟷ A, (10) (CBA x CBA/H-T6)F_1 ⟷ DBA/1, (8) (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, (2) C57BL/6 ⟷ DBA/1, and (13) C57BL/6 ⟷ A mice. [Thymus composition was determined at the time of sacrifice using the trypan blue dye exclusion assay. Coat color phenotype was estimated visually at weaning. Data were taken from Table 22.]


21
14
7
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1


21
14
7
0
No. of Mice
0 10 20 30 40 50 60 70 80 90 100
%STRAIN 1

of coat pigmentation in mouse aggregation chimeras, with the exception of solid color mice which form a peak at each end of the distribution.

Table 25 summarizes the data collected on the mice which have been analyzed for chimeric drift and spleen-thymus discordance. As stated earlier (see Discussion, Section A) deciding what constitutes chimeric drift is not a simple problem since the error of individual determinations can vary between $\pm$ 10-20%. Even though most of the data on chimeric drift was good enough to meet the $\pm$ 20% criterion, a $\pm$ 40% criterion was chosen to be absolutely sure of the results.

The situation with respect to spleen-thymus discordance is much simpler, since the error in these two types of determination is always $\leq$ 10%. Therefore, only one criterion, $>$20% difference in the spleen and thymocyte compositions has been used to enumerate the mice with spleen-thymocyte discordance in Table 25.

The most interesting aspect of the data shown in Table 25 is that 11 out of 14 mice or 79% which show spleen-thymus discordance also showed chimeric drift of their peripheral white blood cells earlier in their lives, when using a $\geq$ 20% criterion. However, using $\geq$ 40% criterion, 4 out of 14 mice or 29% showed the same phenomenon. Only one mouse (159) showing spleen-thymus discordance also showed a significant change in hemoglobin composition. Allophenic mice, therefore, apparently fall into two separate classes: those with stable immune and hematopoietic systems and those with unstable immune and hematopoietic systems. The spleen-thymus discordance in these mice could be either the cause or the result of the

chimeric drift of peripheral white blood cells. It is doubtful this relationship extends to the red blood cell population since only 1 out of 14 mice which showed spleen-thymus discordance also showed significant drift of hemoglobin. If this were true, then it would be highly probable that drift in the two cell populations in adult mice is under the influence of independent mechanisms. However, more mice would have to be examined to verify this observation since a number of mice showing hemoglobin drift were not analyzed for spleen-thymus discordance.

The apparent lack of relationship between hemoglobin drift and spleen-thymus discordance does not exclude the possible influence of the thymus on red blood cell formation and development. Experiments performed by Wiktor-Jedrzejczak et al. (1977) have shown the required presence of a theta-sensititve cell in bone marrow, thymus and spleen for the normal differentiation of stem cells along the erythroid pathway. Their studies indicate that the theta-sensitive regulatory cell (TSRC), shown not to be of hematopoietic stem cell origin, confers a stimulatory action on hematopoietic stem cells resulting in their differentiation into red blood cells. This suggests that deficiencies in either the hematopoietic stem cell or TSRC or both might be one of the mechanisms responsible for some of the genetically determined anemias and stem cell deficiencies seen in certain mouse strains and man.

C. Immunological Tolerance in Allophenic Mice

1. RNA synthesis in mouse spleen cells as a probe of antibody mediated cytotoxicity

Assays for antibody mediated cytotoxicity can be categorized into five classes based upon the method used to distinguish live cells from dead cells. In the dye exclusion assay (Gorer and O'Gorman, 1956; Terasaki and McClelland, 1964; Walford, et al., 1964; Mittal et al., 1968; Amos et al., 1969) the cytotoxic effects of antibody and complement are measured by the failure of dead cells to exclude vital dyes such as trypan blue or eosin Y. Quantitation of cells is determined by microscopic examination of test samples. A variation of the dye exclusion assay is the use of a fluorescent marker (Edidin, 1970; Brawn et al., 1975). In this assay, ethidium bromide is excluded from viable cells but rapidly enters damaged cells, in which it fluoresces. Cytotoxicity is evaluated using a commercially available fluorometer.

A second class of cytotoxic assay is the dye inclusion assay. In the procedure of Filman et al. (1975), target cells, sensitive to antibody-dependent complement-mediated cytotoxicity, undergo an intracellular delocalization of the supravital stain, neutral red. The results are determined by visual enumeration of cells which have accumulated the dye. A similar marker inclusion assay is the fluorochromasia cytotoxicity assay (Rotman and Papermaster, 1966; Bodmer et al., 1967; Watanabe et al., 1971). This assay is based upon the observation that free

fluorescein, produced by intracellular esterase mediated hydrolysis of fluorescein diacetate, is retained by live cells but released from dead cells. Cell viability is determined by counting the number of fluorescent cells in a sample.

In the third category, the colony inhibition assay (Hellström and Sjögren, 1965; Hellström and Hellström, 1971) target cells are grown in petri dishes or microtest plates prior to treatment with antibody and complement. Cells showing no sensitivity to the antiserum are enumerated by visual examination of cells or groups of cells forming colonies.

The fourth category, represented by radioactive marker release assays, involves a pre-assay incubation step in which the radioisotope is concentrated intracellularly. ^{51}Cr-chromate (Sanderson, 1964; Wigzell, 1965; Holm and Perlmann, 1967; Brunner et al., 1968) or ^{86}Rb-chloride (Chollet et al., 1974) are the most commonly used labeling materials. Target cells are then incubated with antibody and complement, and a cytotoxic index determined by the activity released by the test sample divided by total activity released by control cells.

We have developed a new cytotoxicity assay for use on samples of resting mouse spleen cells which contain a large proportion of dead cells due to previous biochemical and immunological treatments. The assay was developed so that it would be refractory to interference by large numbers of dead cells, not require the prelabeling of target cells, be reasonably rapid, be independent of operator subjectivity, and be subject to automation. The dye exclusion and dye inclusion assays could

not be used because of the interference by large numbers of dead cells. The colony formation assay could not be used because it requires a lengthy culturing period. The radioactive marker release assays were not suitable because of the requirement for the prelabeling of the target cells.

The assay developed falls into a fifth category of cytotoxic assays which takes advantage of the fact that live cells will incorporate small molecular weight radioactive precursors into high molecular weight products, whereas dead cells will not. One such assay, which measures the incorporation of [^{3}H] thymidine into DNA was first reported by Klein and Perlmann (1963), and later automated, using a MASH (multiple automated sample harvester) by Knudsen et al. (1974). This DNA synthesis assay procedure meets all the criteria set forth above, except the length of time required. In order to measure DNA synthesis the cells must be undergoing mitosis, and this requires mitogenic stimulation of the spleen cell culture, followed by a several day culture period. In our assay procedure, the basal level of RNA synthetic activity was high enough in resting mouse spleen cells to use the incorporation of [^{3}H]-uridine into RNA as an index of the viability of a given cell population.

Cooper and Rubin (1965), in a series of experiments with resting lymphocytes, have demonstrated that incorporation of [^{3}H]uridine increases over a period of 4-6 hours and then reaches a stable state beyond which

incorporation decreases. Incorporation into high molecular weight product in their studies was linear up to a 5-hour period with no change in the RNA content of the culture during this time. This observation is in agreement with our findings (Figures 10 and 11).

The different classes of RNA synthesized by resting lymphocytes have been previously summarized by Cooper (1971). Briefly, ribosomal RNA, which constitutes the largest pool of RNA in the cell, is turned over quite slowly with a half life on the order of days. It has been estimated that rRNA represents only 2-4% of the total RNA synthesized in the resting cell at any one time. A nonmethylated heterogenous nuclear RNA with a half life in minutes represents 90% of the RNA synthetic activity of the resting cell. The majority of the heterogenous RNA is, in fact, not used but is turned over by the cell (Jelinek et al., 1974). It has been suggested that a portion of the heterogenous RNA is a precursor of messenger RNA. This conclusion is based on its DNA-like base composition, which contains a poly(A) sequence at its 3' end. Heterogenous RNA also shares sequences with mRNA which codes for polypeptide chains (Jelinek et al., 1974). Messenger RNA is thought to represent the processed form of heterogenous nuclear RNA which is transported to the cytoplasm. Cytoplasmic transfer RNA and small molecular weight precursor molecules are turned over at moderately rapid rates and have a half life on the order of 1-2 hours. The types of RNA synthesized during the 5-hour labeling period in our system will have to be confirmed.

Each of the currently available methods for evaluating antibody-mediated cytotoxicity has advantages and disadvantages. Among the commonly used assays, the trypan blue assay has the advantages that it is inexpensive, fast, and requires few manipulations. The disadvantages are the possibility of operator subjectivity, a limit to the number of samples that can be analyzed by a single operator, and the impossibility of evaluating samples when a large percentage of dead cells are present. The ^{51}Cr release assay has the advantage of being objective, but has the disadvantages of requiring the prelabeling and washing of cells. The DNA synthesis assay has the disadvantage of requiring mitogenic stimulation of the culture, and a long culturing period. Even though a large number of cytotoxicity assays have been described previously, none of the known cytotoxicity assays met all the requirements for our work; no interference by large numbers of dead cells, no prelabeling of target cells, no long culturing periods, and no mitogen stimulation of the cultures.

The presently described RNA synthesis assay does meet all the criteria set forth previously. First, there is no interference by dead cells, so that there is no need to separate dead cells nor red blood cells from the live spleen cell population, since neither of these have measurable levels of RNA synthesis. The RNA synthesis assay works as well as Ficoll-Hypaque isolated lymphocytes, and on thymocytes, as on whole spleen cell suspensions. Second, the procedure does not require the extra manipulation of prelabeling the target cells. Third, the

the procedure can be done in hours instead of days as is required in the DNA synthesis assay. By increasing the specific activity of the [^{3}H]-uridine, the assay time could be cut down even further. Fourth, the assay relies on the counting of radioactively labeled material, so it is independent of operator subjectivity. And finally, the procedure could be easily automated using microtiter plates and an automated cell harvester such as the MASH II (Microbiological Associates) or Titertek (Scatron, Flow Laboratories). The RNA synthesis cytotoxicity assay procedure should be applicable to any resting cells with a high basal level of RNA synthetic activity.

2. Cryogenic storage of lymphocytes

Since 1950, several classes of biological materials have been successfully preserved for experimental and clinical usage. This includes spermatozoa (Sadleir, 1966), mouse embryos (Leibo et al., 1975), cornea (Muller et al., 1964), skin (Lehr et al., 1964), and lymphocytes for immunological studies and antibody mediated cytotoxicity testing (Fotino et al., 1967; Bates and Sell, 1970). This success was largely the result of a group of cryoprotective additives that are mixed with cells prior to freezing. The cryoprotective additives are classified as to whether they permeate the cell (intracellular) or remain outside of the cell (extracellular)(Rowe,1966). Examples of a few extracellular additives include such chemicals as polyvinylpyrrolidone (PVP), polyglycol, dextran, lactose, and sucrose. These cryophylactics work most effectively

with cells that require rapid rates of cooling such as red blood cells. They provide their greatest protection by minimizing the effects of ice nucleation by moderating ice crystal growth (Doebbler, 1966). Intracellular additives include glycerol and dimethylsulfoxide (DMSO). These additives are employed with nucleated cells such as lymphocytes that require slow rates of cooling. It is generally believed that penetrating compounds work by minimizing cell dehydration by acting as solvent (H_2O) substitutes, thereby reducing the hypertonicity of the extracellular fluid that often accompanies freezing (Doebbler, 1966).

Procedures for freezing macro- and micro-quantities of lymphocytes for extended periods of time followed by thawing with a minimum loss of viability have been described elsewhere (Fotino et al., 1967; Bates and Sell, 1970). The procedures generally include adding DMSO at a final concentration of 7% (w/v) to cells prior to freezing at 1° C per minute, followed by rapid thawing and washing of cells with culture medium containing serum.

Our study was undertaken to compare the effects of cryogenic storage on allophenic mousespleen white blood cells and thymus cells that are used as target cells in an antibody-mediated cytotoxicity assay before and after freeze-thawing. Also the effects of freeze-thawing on the basal levels of RNA synthesis in spleen white blood cells were examined.

Data summarized in Tables 9 and 10 show the results of the typing of allophenic spleen white blood cells and thymus cells before and after long term freezing and thawing. First of all, it is seen that there is

no significant difference in the percentage of parental cell types comprising the allophenic mice as determined by antibody-mediated cytotoxicity prior to and after long term storage (1-2 years). This was true for all five combinations of allophenic mice. However, spleen white blood cells from individual allophenic mice (120, 125) occasionally did show a significant difference after the freeze-thawing process. This difference probably reflects cell injury during the thawing cycle of those samples, since large groups of cells were frozen simultaneously under identical conditions. Second, it is seen there is no differential susceptibility of any inbred mouse strain or selective advantage of F_1 hybrids over inbred strains, to the detrimental effects of the freeze-thawing process, since the proportions of cell types remained the same, before and after freezing. Therefore, the percentage of cells nonspecifically dead, probably comprises an approximately equal mixture of both parental cell types, with the exception of two mice (120, 125). Third, thymus cells showed fewer nonspecific dead cells than spleen white blood cells, but differences are not statistically significant. Although no one mechanism can account for the nonspecific cell death in the freezing and storage of lymphocytes, a number of factors have been implicated in cell injury. These include the rates of cooling of specimens before and after the initiation of freezing, the formation of ice crystals, their size, location and rate of growth, cellular dehydration as a result of poor distribution of cryoprotective additive, the concentration of solutes, particularly electrolytes, and membrane damage during the

thawing process. The type of vial, whether glass or plastic, had no effect on cell viability. However, the plastic Provials (Cooke Laboratory Products) had an added advantage over glass containers, during cell thawing, in that aerosol formation was minimized and the shatterproof property prevented leakage of the contents of the vials.

No significant levels of RNA synthesis were detected in frozen-thawed lymphocytes. As a result, these cells were unsuitable for usage in assays measuring basal levels of RNA or DNA synthesis (e. g., RNA synthesis assay) as an index of cell viability. Similar results for bone marrow cells slowly frozen in liquid nitrogen have been reported by Rowe (1966). Therefore, frozen cells were found to work best as target cells in dye exclusion assays (E.g., trypan blue dye exclusion assay). Whether this low detectable level of RNA synthesis is the result of the freeze-thawing process or a function of the length of time in storage is not known.

3. The absence of serum blocking factors in allophenic mice

Whether self tolerance in allophenic mice is the result of a central failure of the immune system (Billingham et al., 1954; Burnet, 1959; Jerne, 1971) or is due to some active peripheral mechanisms (Hellström et al., 1971; Allison, 1971; Voisin et al., 1972), has been a subject of long unresolved debate. Mintz and Silvers (1967) first designated tolerance in allophenic mice as being "intrinsic" rather than being "acquired" and proposed that during development reactive clones to the

composite chimera's own tissue were eliminated. Similar observations supporting "clonal elimination" have been reported by other investigators (Meo et al., 1973; Festenstein et al., 1975; von Boehmer et al., 1975a; Barnes, 1976). Contrary to these observations, other workers in the field have presented evidence in favor of serum blocking factors and suppressor cells (Wegmann et al., 1971; Phillips et al., 1971; Phillips and Wegmann, 1973).

Our study fails to confirm the presence of blocking factors in the serum of allophenic mice. A summary of the results can be seen in Tables 26, 27, 28, 29, 30, 31, 32, 33, and 34. The criterion for evaluating blocking activity in the antibody-mediated cytotoxicity assay was defined as a significant difference, at a minimum of two serum dilutions between samples, pretreated with allophenic mouse serum or normal mouse serum, as determined by a Student's "t" test. It can be seen in Table 26, that five serum samples from allophenic mice 105, 107, 120, 138, and 141 showed a significant difference from normal mixed parental serum when tested with their own spleen cells in the trypan blue dye exclusion assay. However, only two serum samples (138, 141) out of 26 samples tested or 8% give serum blocking activity at two serum dilutions. Serum samples from mice 105, 107, and 120 showed significantly higher killing than normal serum controls. This difference was not seen in any of the five mice when the source of the target cells were thymocytes (Table 28). Furthermore, it can be seen in Tables 27 and 29 that serum blocking activity was completely eliminated by testing the same samples across serum dilutions. These data demonstrate that allophenic mouse serum has no blocking effect

of antibody-mediated cytotoxicity on allophenic spleen white blood cells. This was true for mice showing a wide range of chimerism of the immune system (Tables 27and 29). In addition, no relationship was seen between the absence of serum blocking activity and the phenomena of chimeric drift and spleen-thymus discordance.

Analysis of serum blocking activity using allophenic mouse serum and parental type spleen white blood cells gave similar results (Tables 30, 31, 32, 33, and 34). Of the 90 duplicate cytotoxicity tests performed on 45 allophenic mouse serum samples, 7/90 or 8% were significant at the 0.05 level while 2/90 or 2% were significant at the 0.01 level. It could be argued that serum blocking may have been present but repeated freeze-thawing and heat inactivation of allophenic serum samples destroyed their protective properties. However, in other studies (Wegmann et al., 1971; Phillips et al., 1971) where allophenic mouse sera received similar treatment, serum blocking activity was reported.

This reported absence of serum blocking activity is not in accordance with the studies of Hellström (Hellström et al., 1971) and Wegmann and Phillips (Wegmann et al., 1971; Phillips et al., 1971). In the studies of Hellström, mice made tolerant by the neonatal transfer of F_1 generation cells were reported to possess self-reactive clones whose sera could block the killing of donor strain fibroblasts in the microcytotoxicity (MC) test. However, in subsequent studies by Beverley (Beverley et al., 1973) such mice have been shown to be characterized as being partially tolerant, due to insufficient dosage of F_1 cells in

the Hellström tolerance inducing procedure. As a result, only partially tolerant states have been characterized by the presence of blocking factors. On the other hand, allophenic mice have been shown to be healthy and long-lived and readily accept skin grafts from either of the two component strains indicating full tolerance (Mintz and Silvers, 1967). Wegmann and his colleagues (Wegmann et al., 1971) demonstrated that lymph node cells from C57BL/6 ⟷ C3H/He or (CBA x C57BL/6)F_1 ⟷ SJL mice could react against parental cell lines in the (MC) test. Phillips et al. (1971) similarly showed that C57BL/10 ⟷ SJL lymphoid cells could react against cells with the corresponding parental strain antigens in mixed lymphocyte culture (MLC). Furthermore, this in vitro reactivity of chimeric cells to parental cells could be blocked by sera from such mice (Wegmann, et al., 1971; Phillips et al., 1971). Serum blocking factor was characterized as an antibody molecule with specificity for the allelic products of the H-2 region (Phillips et al., 1971). However, in this study, sera from allophenic mice did not protect target cells from the toxic action of antiserum and complement. Unlike prior tolerance studies on allophenic mice (Wegmann et al, 1971; Phillips et al., 1971), where serum samples from three or four mice of one or two parental combinations were pooled, our study tested 52 different unpooled serum samples from six different parental combinations on a variety of target cells. In addition, unlike the Wegmann and Gilman studies, the immune system composition of individual mice was characterized at the time of serum collection.

Other investigations failing to show evidence of serum blocking factors in allophenic mice include Meo (Meo et al., 1973), Festenstein (Festenstein et al., 1975) and von Boehmer (von Boehmer et al., 1975a). Meo and Festenstein in separate studies demonstrated allophenic mouse serum or cells could not prevent reactivity of parental cells in one way MLC. Similar results were reported by Ceppellini (1971) and his associates in the case of tetragametic human chimeras. von Boehmer (1975a) and his group working with tetraparental bone marrow chimeras produced by injecting lethally irradiated F_1 hybrid mice with relatively high numbers of T cell depleted bone marrow from two allogeneic parental strains reported the absence of any reactive cells in MLC and cell-mediated lympholysis (CML) of chimeric origin to host cells. In addition, no suppressor cells could be demonstrated in either system and serum blocking factors could not be found. These results are in direct conflict with the previous studies of Phillips and Wegmann (1973). They suggested that chimeric spleen cells insensitive to antitheta serum could block MLR between the two parental cell types, but not between unrelated strains.

In summary, our study fails to confirm the presence of serum blocking factors in the sera of allophenic mice as detected by antibody-mediated cytotoxicity. Likewise, other investigators have failed to detect any blocking activity of chimeric serum in MLR and CML (Meo et al., 1973; Festenstein et al., 1975; and von Boehmer et al., 1975a) or in the <u>in vivo</u> local graft-versus-host assay (Barnes and Graham, 1976).

Presumably, the presence of serum blocking factors could be detected in any or all of the assays. In their experiments, Phillips and Wegmann (1973) suggested the levels of serum blocking factor varied between different strain combinations of allophenic mice. In this study, no blocking activity was detected from 52 different serum samples from six different parental combinations. Likewise, the possible role of suppressor cells has not been substantiated (Barnes, 1976). Although no interpretation of tolerance in allophenic mice can be absolute, collectively these observations are in strong agreement with the concept of a central failure of the immune system.

D. Conclusion

A basic goal of this research was to explain the phenomena of chimeric drift and spleen-thymus discordance in terms of the presence of serum blocking factors which are characteristic of "graft versus host" reactions and incomplete states of tolerance. The results presented in this dissertation are unequivocable. Neither stablenor unstable allophenic mice have detectable serum blocking activity as determined by antibody mediated cytotoxicity testing. This observation is consistent with the results of other workers (Meo et al., 1973; Festenstein et al., 1975; von Boehmer et al., 1975a; Barnes and Graham, 1976) who similarly have failed to detect serum blocking activity or suppressor cells in allophenic mice. Thus, the overall picture that emerges from these observations is that chimeric

drift in the two cell populations is probably the response of two independent control mechanisms and the mechanisms involved are not the same as those responsibile for maintenance of tolerance. This conclusion is supported by three independent studies. First, it was seen in this study that significant changes in the red blood cell populations of allophenic mice occurred independently of changes in the white blood cell populations and that 79% of the mice showing spleen-thymus discordance also showed peripheral white blood cell drift. These observations are consistent with the investigations of Berntson (1978) who observed only a slight correlation of the IgG2a allotype marker with hemoglobin composition of allophenic mice and, therefore, concluded that the red blood cell and white blood cell populations must be under separate regulatory mechanisms. Second, it was seen in both cattle (Stone et al., 1964) and marmoset (Genozian et al., 1969) chimeras, that spontaneous change in the proportion of the red blood cell populations with time could occur with no apparent breakdown of tolerance in these animals. This closely resembles the results seen here with primary chimeras. Finally, Gandini (Gandini et al., 1968) and his colleagues investingating x-inactivated mosaics, observed that granulocytes and erythrocytes appear to be derived from a common pool of precursor cells while lymphocytes were derived from a different precursor pool. Both precursor pools appeared to be regulated by separate controls. Although chimeric drift in the peripheral white blood cell population appears to be the cause or the result of spleen-thymus discordance, it is still possible that both phenomena are independent

of each other. An important question which remains to be answered is whether the lymphomyeloid tissues arise from a common stem cell (Gornish et al., 1972) or whether certain groups of immune tissues are derived independently (Ford et al., 1975).

The value of the investigation of tolerance in allophenic mice is great. Understanding the mechanism by which genetically distinct cells coexist in an allophenic animal may help us understand the normal mechanism of tumor development and regression. It is the hope of the author that the data collected and presented herewithin may directly or indirectly help in the future understanding of how cells interact with each other.

V. SUMMARY

One hundred five allophenic mice of nine different types [C57BL/6 ⟷ (A x SJL)F_1, DBA/1 ⟷ (A x SJL)F_1, DBA/1 ⟷ A, (CBA x CBA/H-T6)F_1 ⟷ DBA/1, (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, (CBA x CBA/H-T6)F_1 ⟷ A, C57BL/6 ⟷ DBA/1, C57BL/6 ⟷ A, and C57BL/10 Sn ⟷ A] were produced for characterization and quantitation of their hematopoietic and(or) immune systems and for tolerance studies. Techniques were developed to examine erythrocyte, peripheral white blood cell, spleen white blood cell, and thymocyte compositions of the mice. This approach was achieved by using chemical modification with cystamine dihydrochloride and polyacrylamide gel isoelectric focusing (PAGIF) for the separation of the different hemoglobin types, and antibody-mediated cytotoxicity for quantitation of spleen white blood cells, peripheral white blood cells and thymocytes. Mice were analyzed for changes in their peripheral white blood cell and hemoglobin compositions at several time points in their lives. Spleen white blood cells, peripheral white blood cells and thymocytes were analyzed at the time of sacrifice. It was found that the red blood cell and white blood cell populations of 21 of the 85 mice examined showed significant changes termed "chimeric drift" in one or the other or both of these parameters. These 21 mice were classified as unstable chimeras, as opposed to the 64 stable chimeras which showed no apparent drift. There was an excellent correlation of the peripheral white blood cell and hemoglobin composition of the stable chimeras. However, the

unstable chimeras showed little or no correlation of these two markers. At the time of sacrifice, the immune system of 93 allophenic mice was studied. All eight types of mice which were characterized [C57BL/6 ⟷ (A x SJL)F_1, DBA/1 ⟷ (A x SJL)F_1, DBA/1 ⟷ A, (CBA x CBA/H-T6)F_1 ⟷ DBA/1, (CBA x CBA/H-T6)F_1 ⟷ C57BL/6, (CBA x CBA/H-T6)F_1 ⟷ A, C57BL/6 ⟷ DBA/1, and C57BL/6 ⟷ A] were found to be true chimeras since the vast majority of cells in these chimeric animals had only one or the other parental types of surface antigens. It was seen that the proportions of the parental thymocytes (and coat color, spleen white blood cells and peripheral white blood cells) in all eight types of allophenic mice approximates an uniform distribution, with each proportion almost occurring at an equal frequency. The correlation of the blood with the spleen composition for each of the eight types was excellent for all mice. However, only two types of mice, (CBA x CBA/H-T6)F_1 ⟷ DBA and C57BL/6 ⟷ A, showed a good correlation of the thymus composition with either the blood or the spleen composition. Further analysis of individual mice of the eight types revealed that 11 out of 14 mice which showed spleen-thymus discordance also showed chimeric drift of their peripheral white blood cells ($\pm$ 20% criterion) earlier in their lives. On the other hand, only 1 out of 14 mice with spleen-thymus discordance also showed chimeric drift of the red blood cell population.

In tolerance studies, serum samples from 52 unstable and stable allophenic mice of similar combinations were analyzed for serum blocking activity using an antibody-mediated cytotoxicity assay. The target cells consisted of frozen-thawed allophenic mouse spleen white blood cells, or

thymocytes and freshly collected parental strain spleen white blood cells. Serum treated frozen-thawed cells were typed using the trypan blue dye exclusion assay while serum treated freshly collected cells were typed by measuring basal levels of RNA synthesis in live cells as a probe of antibody-mediated cytotoxicity. It was found that neither 14 unstable or 38 stable allophenic mice had serum blocking activity as detected in these assays. In addition, 10 mice which showed spleen-thymus discordance also failed to show the presence of blocking factors in their serum. Although no interpretation of tolerance in allophenic mice can be absolute, these observations are in agreement with the concept of a central failure of the immune system.

In conclusion, chimeric drift in the red blood cell and white blood cell populations is probably the result of two independent control mechanisms and the mechanisms involved are not the same as those responsible for the maintenance of tolerance. The spleen-thymus discordance in these same mice could either be the cause or the result of chimeric drift or be totally independent of this phenomenon. Explanation of the immune system of allophenic mice suggests that all the lymphomyeloid cells could have a common origin in embryogenesis, and then diverge due to the differential proliferation of one cell type or the other in the adult allophenic animal. These studies have laid the groundwork for further experiments aimed at the elucidation of the mechanism of tolerance in allophenic mice.

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